DORSAL-VENTRAL RETINAL PATTERNING: GENETIC MECHANISMS FOR THE DEVELOPMENT OF A TOPOGRAPHIC MAP

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Topographic connections of retinal axons with their brain targets allow us to perceive a spatially organized image of the visual world. Achieving this precise retinotopic axon targeting during development first requires the patterning of origin (retina) and target (brain) tissues along anterior-posterior (A-P) and dorsal-ventral (D-V) axes, such that individual cells of both tissues acquire a molecularly specified positional identity.

The research presented in my dissertation addresses the processes involved in patterning the dorsal-ventral axis of the retina and forming dorsal-ventral retinotopic projections. First, I studied what tissues and signals are involved in the initiation of dorsal polarity. I found that the dorsolateral region of the optic vesicle is the first domain to upregulate dorsal-specific transcription factors. My expression studies, combined with my fate map experiments, suggested that these dorsolateral optic vesicle cells continue expressing dorsal markers throughout optic vesicle morphogenesis, and eventually contribute to dorsal and central retina. I also showed that the BMP family gene *gdf6a* is necessary for initiation of dorsal retinal fate. Importantly, I found that *gdf6a* acts early during optic vesicle evagination (10-12 hpf) when it is expressed in the extraocular head ectoderm overlying the region of dorsolateral optic vesicle that first expresses dorsal-specific transcription factors. Finally, I showed that the *bmp2b* gene is also necessary for dorsal retinal fate initiation, acting upstream of *gdf6a*.

Secondly, I performed a forward genetic screen to search for novel genes that specify the dorsal-ventral axis of the retina. By screening for mutants with altered expression of D-V markers, I identified a novel gene, *bigtop*, required for dorsal-ventral retinal patterning, eye development, and retinotectal projections. This mutation was mapped to a four-megabase region on chromosome two.

My studies addressed the following question: how is the retina patterned early during development in order to specify retinal ganglion cells with positional fate? Altogether, my studies advanced the field of D-V retinal patterning by identifying an extraocular dorsal retinal initiation signal, uncovering the role of *bmp2b* in dorsal retinal initiation, and identifying a novel mutant necessary for D-V patterning.

For Chi-Bin

Who taught me to follow my heart in the face of any obstacle

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CHAPTER 1

INTRODUCTION

Summary

Topographic axon connections are prevalent in all sensory systems, and are critical for sensory information processing (Luo and Flanagan, 2007). How do axons achieve this precise architecture during development? Development of topographic projections requires that source and target tissues are first patterned by gene expression in precise domains (McLaughlin et al., 2003). Next, axon guidance molecule gradients within each tissue direct axons to their appropriate targets (McLaughlin and O'Leary, 2005). I used the well-characterized retinotectal projection of zebrafish to study the gene networks responsible for tissue patterning and formation of topographic projections of retinal ganglion cells (RGCs). Specifically, my research presented here tested the roles of genes expressed along the dorsal-ventral axis of the retina.

Zebrafish is an excellent model for my research, primarily because zebrafish embryos are clear and develop externally, allowing visualization of eye formation and axonal targeting *in vivo*. Zebrafish are genetically tractable, and amenable to both forward and reverse genetic manipulation. Furthermore, while there is a high level of gene conservation between vertebrates, recessive mutations that are embryonic lethal in mice often are not lethal in zebrafish prior to eye development and retinotopic map formation, thus allowing me to analyze the effect of these of mutations on visual system

development. Finally, zebrafish eye development occurs rapidly (Figure 1) (Kwan et al., 2012), and retinotectal mapping is precise from an early stage (Stuermer, 1988).

The research presented herein is driven by a broad question: what molecules and mechanisms are involved in patterning the dorsal-ventral (D-V) axis of the retina and organizing topographic retinotectal projections? To address this question, I analyzed tissues and genes important for initiating patterning in the dorsal retina, and screened for novel genes involved in specifying the D-V retinal axis. Thus, my dissertation work investigates how the retina is patterned along the D-V axis in order to establish accurate topographic retinotectal connections.

This work has broad relevance. Retinal patterning mechanisms are conserved through vertebrate evolution, from fish to humans. Therefore an understanding of the genetic pathways that underlie the patterning and topographic axon projections of the retina in the zebrafish will increase our understanding of eye development and axial patterning mechanisms in all vertebrates. Ultimately, these studies could lead to treatments for developmental eye diseases such as coloboma and microphthalmia.

Axial Patterning and the Retinotopic Map

The projections of RGC axons to the optic tectum (or superior colliculus in mammals) form a retinotopic map, in which axons of neighboring RGCs project to neighboring areas of the optic tectum (Figure 2A), thus providing a continuous view of the visual world in the brain (Figure 2B). This architecture is required for accurate processing of visual information. For these projections to develop, retinal (origin) and tectal (target) tissues are first patterned along perpendicular axes. Both the retina and tectum are patterned along dorsal-ventral (D-V) and anterior-posterior (A-P) axes, as

evidenced by the numerous genes expressed in D-V and A-P domains. Polarized gradients of gene expression encode cells of each tissue with a molecularly specified positional identity, thus allowing axons of source cells to identify their targets (McLaughlin et al., 2003; Harada et al., 2007).

One conserved tissue patterning mechanism for establishing positional identity of cells within a tissue is for morphogens, diffusible signaling molecules, to initiate polarity by forming a concentration gradient that is high at one end of the axis and low at the other (e.g. high dorsal and low ventral expression along the D-V axis). Importantly, these morphogen signals can arise from a neighboring tissue source, and diffuse into the tissue of interest to induce positional fate, as is the case in the neural tube, which is patterned by a gradient of SHH from the underlying notochord (Briscoe and Ericson, 2001; Chamberlain et al., 2008). Morphogens then regulate the expression of transcription factors in overlapping domains within the tissue. Transcription factors expressed in polarized domains can in turn regulate polarized expression gradients of axon guidance molecules (Figure 3). Ultimately, axon guidance receptors on growth cones can mediate topographic targeting by instructing axons to navigate toward attractive and away from repulsive ligands in their environment. This allows them to take a path adjacent to, but distinct from neighboring axons that express a slightly different complement of receptors.

My studies analyzed the genes and mechanisms involved in tissue patterning along the dorsal-ventral axis of the retina, specifically focusing on the dorsal half of this axis. Along this axis, dorsal RGCs target to the contralateral ventral (lateral) tectum and ventral RGCs to the contralateral dorsal (medial) tectum. Therefore, RGC axons create a projection of the visual world onto the contralateral optic tectum (Figure 4). I addressed

both initial tissue induction aspects of forming this dorsal-ventral topographic map (Chapter 2), as well as carried out a genetic screen to identify new molecules important for retinal dorsal-ventral patterning (Chapter 3).

Current Models of Dorsal-Ventral Retinal Patterning

Past research has identified several genes involved in retinal dorsal-ventral patterning and topographic map formation (Baier et al., 1996; Koshiba-Takeuchi et al., 2000; Sakuta et al., 2001; Zhang and Yang, 2001; Baumer et al., 2002; Mann et al., 2002; Sasagawa et al., 2002; Liu et al., 2003; Liu et al., 2004; Murali et al., 2005; Muto et al., 2005; Behesti et al., 2006; Sakuta et al., 2006; Asai-Coakwell et al., 2007; French et al., 2007; French et al., 2009). In particular, specification of the dorsal axis is thought to be regulated by dorsally expressed retinal BMP morphogens, which in turn upregulate the expression of dorsal *tbx* T-box transcription factor genes. These Tbx transcription factors then upregulate dorsal expression of EphrinB axon guidance molecules to specify dorsal axon targeting (McLaughlin et al., 2003). In support of this model, *Bmp4* knockout mice have no *Tbx5* expression at E9.5 (Murali et al., 2005). Bmp4 gain of function experiments, and loss of function of *Bmp* receptors also implicate Bmp regulation of *tbx5* in the dorsal retina (Murali et al., 2005; Behesti et al., 2006). Gain-of-function experiments in the chick indicate that TBX5 positively regulates *EPHRINB* expression and perturbs retinotopic axon projections (Koshiba-Takeuchi et al., 2000). Furthermore, gain-of-function and dominant negative experiments in *Xenopus*, and loss-of-function in the mouse, indicate that *EphrinB2* plays a role in topographic mapping (Mann et al., 2002; Thakar et al., 2011).

Ventrally, morphogen genes *Shh* and the Bmp antagonist *Ventroptin* are thought to regulate expression of the homeobox transcription factor gene *Vax2* (Sakuta et al., 2001; Zhang and Yang, 2001; Barbieri et al., 2002). In turn, *Vax* can regulate expression of *EphB* axon guidance receptor genes (Peters, 2002). Axon guidance molecule genes important ventrally likely include *EphBs*, *Ryk*, and *semaphorins* (Hindges et al., 2002; Liu et al., 2004; Schmitt et al., 2006).

Additions to the Current Model of D-V Patterning:

Role of an Extraocular Tissue

We speculated that specification of the dorsal retina had additional layers of complexity. Many genes with proposed importance in dorsal retinal patterning have only been tested using gain-of-function approaches, and my loss-of-function studies in zebrafish did not corroborate the results of several of these misexpression studies. The current model posits that *Bmp4* within the retina is the first gene to specify dorsal fate. However, the positional information to activate *Bmp4* expression in this specific domain is not known. We propose that an extraocular factor is necessary for initiating dorsal fate. Furthermore, my data and that of others, indicate that *bmp4* does not initiate dorsal-specific gene expression in zebrafish, whereas the Bmp family member *gdf6a* plays an important role in initiation of dorsal fate (Asai-Coakwell et al., 2007; French et al., 2007). Additionally, while *BMP2* in the chick has been shown to be necessary for maintenance of expression of many dorsal-ventral axis genes, it has been shown that it is not required for initiation of these genes. Indeed, the continuing expression of all known patterning genes from early to late stages of eye development suggests that feedback mechanisms may be important for maintaining gene expression; however distinguishing initiation

versus maintenance roles of dorsal genes has not been a focus of previous studies. Finally, four *tbx* genes in addition to *tbx5* are expressed in dorsal retina in zebrafish, and progressively are refined to more dorsal domains over time. Yet the cause of this domain refinement and the roles of *tbx* genes in D-V patterning are unknown.

Because genes with known roles in D-V patterning act at different stages of development, we propose that dorsal retinal development could be viewed in three stages: 1) initiation, 2) maintenance, and 3) refinement of dorsal fate, and that many genes likely act during each of these stages (Figure 5). In studying the genetic mechanisms of dorsal retinal patterning and topographic projections, I have enhanced our understanding of D-V patterning by differentiating between initiation, maintenance and refinement stages of the process.

Initiation of D-V Patterning

It is widely accepted that both morphogens and transcription factors play roles in specifying dorsal retinal polarity. However, the genes necessary for initiating dorsal character have not been identified prior to this work. We hypothesized that the initiation signal for specification of dorsal fate is expressed external to the eye field, in a location allowing it to induce dorsal identity within the retina (Figure 5). The first known expression of retinal dorsal identity in zebrafish is the expression of the T-box transcription factor genes, *tbx2a* and *tbx5a*, in restricted domains. The primary candidates for regulation of *tbx* genes, and thus initiation of dorsal identity, are the morphogens of the bone morphogenetic protein family (Bmps). At least five *bmp* genes mark the dorsal domain of the zebrafish eye at 24 hpf—*bmp2a bmp2b*, *bmp4*, *bmp7b* and *gdf6a*, and are likely candidates for dorsal initiation and maintenance signals. However, these genes are

not expressed within the retinal field until after initiation of dorsal fate, as evidenced by the expression of dorsal-specific *tbx* transcription factor genes. I asked which of the candidate *bmp* morphogen genes are expressed in a place and time that they could initiate the prospective dorsal retina? To answer this question, I first analyzed which cells are prospective dorsal in the early optic vesicle.

Morphogenesis of the Dorsal Retina

The original location of prospective dorsal cells within the early optic vesicle has not been clearly described (Woo and Fraser, 1995; Li et al., 2000; England et al., 2006). In *Xenopus*, fate maps indicate that cells of the medial optic vesicle become ventral retina, and suggest that cells of the lateral optic vesicle may be prospective dorsal (Eagleson et al., 1995). I performed fate-mapping studies indicating that prospective dorsal cells are located within the lateral optic vesicle at 12 hpf, as suggested in *Xenopus*. This also matches the 12 hpf expression domains of *tbx2a* and *tbx5a*, the earliest dorsal-specific transcription factor genes known to be expressed in restricted domains within the optic vesicle (Figure 5, Initiation time point, blue). Furthermore, this is consistent with fate map data, showing that a segment of the 12 hpf dorsolateral optic vesicle maps to the 24 hpf dorsal domain (Kwan et al., 2012). Together, these data suggest that dorsal retinal initiation signals should be located adjacent to the dorsolateral optic vesicle, in the non-neural ectoderm adjacent to and overlying the optic vesicle, and be expressed just prior to 12 hpf (Figure 5, Initiation, orange).

Gene Expression of Candidate Dorsal Morphogen Initiators

Bmps are known to be important for establishing dorsal retinal fate (Kishimoto et al., 1997; Murali et al., 2005; Behesti et al., 2006; Sakuta et al., 2006), however their role in initiating dorsal polarity had not been specifically analyzed prior to this work. I performed expression studies to determine which of the dorsally expressed *bmp* genes are expressed in the right place at the right time to act as dorsal initiators. I showed that two of our candidate genes, *bmp2b* and *gdf6a* are expressed in the lateral head ectoderm at 11-12 hpf, thus fulfilling our criteria to be dorsal initiation signals.

Initiation and Maintenance of Dorsal Patterning in Dorsal Morphogen Mutants

The gene expression cascade that establishes dorsal fate likely includes signals that are necessary for both initiation and maintenance of dorsal patterning genes. I analyzed both initiation and maintenance time points of dorsal-specific gene expression within the eyes of mutants, to test the required roles of the mutated genes for specifying dorsal identity.

Gdf6a

A *gdf6a* allele, *s327*, was identified in a forward genetic screen for mutants with defects in visual background adaptation (Muto et al., 2005). Dorsal RGCs in *gdf6a*^{s327} homozygotes misproject their axons through the dorsal optic tract along with ventral RGCs, and innervate the dorsal tectum, thus leaving the ventral tract and tectum (where dorsal axons normally project) completely devoid of axons. Both the RGCs and tectum appear normal morphologically, (with the exception that the ventral tectum is not

innervated). However, despite the fact that all axons innervate the dorsal tectum, there is no defect in their A-P topographic patterning: anterior RGCs project to the posterior tectum, and posterior RGCs project to the anterior tectum (Muto et al., 2005). These defects suggest that *gdf6a* is specifically involved in imparting dorsal RGC identity. My *in situ* hybridization data, combined with data from other labs (French et al., 2007; Gosse and Baier, 2009) showed that *gdf6a* is necessary for the normal initiation of dorsal patterning genes. Dorsal markers *tbx2a*, *tbx3, tbx5a*, *bmp2b*, *bmp4, bambi, ephrinB1*, *ephrinB2a*, and *raldh2* are either not expressed or greatly downregulated in *gdf6a* mutants. The known ventral RGC marker genes *vax2* and *ephB2* are normally initiated, but later expand to fill most of the retina. Therefore, *gdf6a* is a critical initiation factor for dorsal retinal fate. However the expression pattern of *gdf6a* during dorsal retinal initiation has not been studied previously. Therefore, it is not known whether Gdf6a is acting extraocularly to initiate dorsal fate, or whether Gdf6a is acting within the retina, suggesting yet another extraocular signal for initiating retinal Gdf6a.

Bmp2b

Recent experiments in the mouse have demonstrated that transgenic misexpression of *Bmp2* or the Bmp antagonist *Noggin* has profound effects on dorsoventral retinotopic projections (Plas et al., 2008). However, *Bmp2* knockout mice die at E7-E9, precluding analysis of eye development. In the chick, shRNA knock down of *BMP2* caused loss of dorsal markers and expansion of ventral markers, accompanied by retinotectal projection defects. Yet *BMP2* in the chick is first expressed after the markers it was shown to regulate, and *BMP2* did not affect the initiation of dorsal patterning molecules in this model, only their maintenance (Sakuta et al., 2006).

In zebrafish, *bmp2b* is expressed in the lateral head ectoderm during optic vesicle evagination, and in the dorsal retina beginning at 15 hpf. The zebrafish *bmp2b* mutant allele *tc300* (also called *swirl*) was identified in a large scale forward genetic screen for embryonic phenotypes (Mullins et al., 1996; Kishimoto et al., 1997). *bmp2b*^{tc300} mutant embryos are strongly dorsalized at 12 hpf and die due to yolk lysis by ~15 hpf (Mullins et al., 1996). My dorsal marker analysis in *bmp2b* mutants showed that *tbx5a* and *tbx2a* genes are not initiated, yet the *rx3* homeobox transcription factor gene identifies an eye field in these mutants, and the prospective ventral optic stalk marker *pax2a* is expressed (Thisse, 2001). Therefore, I showed a novel role for *bmp2b*, as a critical initiator of dorsal polarity in zebrafish.

Bmp4

In the mouse, *Bmp4* is the proposed initiator of dorsal retinal fate. *Bmp4* turns on prior to *Tbx5* in the retina, and *Bmp4* knockout mice lack *Tbx5* expression in the retina at E9.5 in the mouse (Murali et al., 2005). This suggests that *Bmp4* knockouts do not initiate dorsal retinal fate. However, these mice die after E9.5 (Winnier et al., 1995), precluding further analysis of gene expression or retinotectal topography. Bmp4 misexpression has been shown to positively regulate *Tbx5* and other T-box transcription factors in the mouse retina (Behesti et al., 2006), again suggesting that *Bmp4* regulates early dorsal gene expression. However, since different Bmps have similar activities in vivo it is hard to interpret the results from these misexpression experiments. Therefore, I believed that loss-of-function experiments in the zebrafish would clarify the role of *bmp4* in eye development.

In the zebrafish, *bmp4* is not expressed in the retina until 14 hpf, 2.5 hours after the initiation of dorsal identity as seen by expression of *tbx2a* and *tbx5a*. Prior to 14 hpf, *bmp4* is expressed anterior to the eye field in the prechordal plate. The zebrafish $bmp4^{st72}$ null mutant was found in a reverse genetic DNA screen from a library of mutagenized F1 males (Stickney et al., 2007). My *in situ* hybridization data showed that this mutant exhibits normal expression of the dorsal marker gene *tbx5a*. Therefore it is unlikely that *bmp4* is required to initiate dorsal polarity in the zebrafish.

One eyed pinhead

The zebrafish *one eyed pinhead* allele, oep^{m134}, was identified in a large scale forward genetic screen in (Haffter et al., 1996). *oep* is an essential Nodal cofactor gene, related to *cripto* (Zhang et al., 1998; Gritsman et al., 1999). *oep* mutants lack anterior axial mesoderm, and therefore never form prechordal plate, one of the tissues arising from this portion of the mesoderm. We used this mutant to assay the effect of loss of prechordal plate on dorsal eye initiation. In the zebrafish, *bmp4* is expressed extraocularly in the prechordal plate tissue, just anterior to the optic vesicle, between 10-14 hpf. We hypothesized that *bmp4* from this tissue may be contributing to dorsal fate initiation. However *oep* mutants lacking prechordal plate and the *bmp4* expression within it show normal initiation of dorsal retinal markers, ruling out this hypothesis.

Novel Genes Involved in Dorsal-Ventral Retinal Patterning

Mutants exist for several candidate genes involved in D-V patterning. Yet our analyses of these mutants indicated that large gaps existed in the understanding of the mechanisms for specifying the dorsal-ventral retinal axis. These gaps were unlikely to be

resolved even after complete analysis of known candidates genes. Therefore, I performed an F3 forward genetic screen to identify novel genes involved in D-V patterning (Figure 6A). Genetic screens are useful for discovering genes that are required for a process of interest in an unbiased fashion, thus complementing our analysis of candidate genes. No previous screens had specifically sought D-V retinal patterning mutants, and previous screens for retinotectal projection phenotypes or other phenotypes had found only three D-V mapping mutants—*nevermind*, *who-cares*, and *gdf6a* (Baier et al., 1996; Muto et al., 2005). Therefore, my novel screen was likely to identify new genes required for dorsal-ventral patterning. I used an *in situ* hybridization cocktail composed of a dorsal and ventral marker – *tbx5a* and *vax2* respectively (Figure 6B), and analyzed F3 clutches for mutants, as seen by changes in the expression pattern of one or both of our D-V marker genes at 24 hpf. With the marker genes used, and the time of our assay (24 hpf), I expected to find mutations affecting all three stages of dorsal retinal identity – initiation, maintenance, and refinement.

Research Summary

My dissertation research addressed the genes involved in patterning the dorsal-ventral (D-V) axis of the retina, a critical step in directing topographic retinotectal projections. I used the zebrafish as a model, and pursued two research questions: a) what genes are important for initiating patterning in the dorsal retina (Chapter 2), and b) what novel genes are involved in specifying the D-V retinal axis (Chapter 3).

Chapter 2

In Chapter 2 I characterized the early events of dorsal retinal patterning and determined the required roles of candidate morphogens for initiation of dorsal polarity. We hypothesized that the initiation signal would arise from an extraocular source. I took a four-part approach to determining what genes are important for initiating dorsal fate, and from where, by analyzing 1) eye morphogenesis, 2) gene expression patterns, 3) pharmacological inhibition of initiation, and 4) dorsal morphogen mutant phenotypes.

Regarding eye morphogenesis, knowing the position of prospective dorsal cells within the eye field just prior to the establishment of dorsal polarity should indicate the probable location of extraocular dorsal initiation signals. I hypothesized that the earliest manifestation of dorsal fate within the retina was the polarized expression of the T-box transcription factor genes *tbx2a* and *tbx5a* within the dorsolateral optic vesicle domain.

I also hypothesized that these cells would migrate during morphogenesis of the eye to populate the dorsal retina. I determined the placement of prospective dorsal cells within the optic vesicle using a Kaede fate mapping approach. Kaede is a photoactivatable fluorescent protein that undergoes an irreversible photocleavage to change from green to red fluorescent protein upon exposure to 405 nm light. I used nls-tagged Kaede mRNA injected into one-cell stage wild-type embryos. I photoactivated cells in the lateral optic vesicle (an area within the *tbx5a* gene expression domain) to photoconvert this area to red fluorescent protein. I then imaged these optic vesicles at one-hour intervals to track the movements of the lateral optic vesicle cells. My data indicated that a portion of lateral cells of the optic vesicle, also expressing the dorsal transcription factor *tbx5a,* undergo morphogenesis to reside in the dorsal optic cup.

I used the selective pharmacological inhibitor of Bmps LDN 193189 to show that Bmps are necessary for dorsal retinal initiation, prior to the first known expression of Bmps within the retina. Inhibition of Bmp signaling with this pharmacological agent from 9.5-13 hpf lead to loss of dorsal retina induction, showing that Bmps are necessary for dorsal fate initiation during optic vesicle evagination. Because they are necessary for initiation prior to their expression in the optic vesicle, this suggested that Bmps are the critical extraocular initiators of dorsal fate.

Initiation genes must be expressed in the right place at the right time to give prospective dorsal cells their identity. Early gene expression of dorsal-specific transcription factor genes *tbx2a* and *tbx5a* is observed in the lateral optic vesicle, and my preliminary fate map data indicated that lateral optic vesicle gives rise to the dorsal optic cup. I therefore expected that extraocular initiation factors for dorsal retinal polarity would be located lateral to the optic vesicle just prior to 12 hpf, in the lateral head ectoderm. Therefore I analyzed which dorsal morphogen genes (candidate initiators) are expressed in the lateral head ectoderm, adjacent to the *tbx*-expressing prospective-dorsal region of the optic vesicle, during evagination. Using *in situ* hybridization, I showed that two of our candidate genes, *gdf6a* and *bmp2b* are expressed in the lateral head ectoderm during evagination, prior to initiation of dorsal-specific *tbx* transcription factor genes in the optic vesicle. Past work (Nguyen et al., 1998) and my data have shown that *bmp2b* is necessary for the specification of non-neural ectodermal tissue during early development. Therefore, I believe that the primary role for Bmp2b in dorsal initiation is early, acting in formation of the extraocular ectoderm tissue. However, I could not rule out another role for this gene during dorsal initiation at the time point of optic vesicle evagination (10-12

hpf). We also showed that *bmp4* is expressed extraocularly at the correct time, in a location anterior to the optic vesicle, the prechordal plate. We use the *one eyed pinhead* mutant *oep*, which lacks prechordal plate tissue, to demonstrate that this tissue, and *bmp4* expression arising from it, is not necessary for dorsal initiation.

Finally, I analyzed the initiation and maintenance of D-V retinal patterning genes in candidate gene mutants. My data identified Gdf6a as a necessary dorsal initiation signal. Dorsal markers are never initiated in *gdf6a* mutants, and ventral markers, while initiated normally, expand over time to fill the majority of the retina. Furthermore, we showed that *gdf6a* is acting from the extraocular ectoderm, overlying the dorsolateral optic vesicle domain of *tbx* expression. Our results show that *bmp2b* is also necessary for dorsal initiation, as dorsal markers are not initiated in this mutant, while ventral markers are expressed in normal domains. I showed that *bmp2b* acts upstream of *gdf6a*, likely to specify the extraocular ectoderm – the critical tissue for expressing the *gdf6a* dorsal initiation signal. *Bmp4* is implicated as an initiator in other models, but my results suggest this is not the case in the zebrafish. Both dorsal and ventral markers are initiated and maintained normally in *bmp4* null mutants.

Chapter 3

I performed an F3 mutagenesis *in situ* hybridization screen to generate and recover new mutations in genes that are functionally necessary for establishing dorsal-ventral retinal polarity. I used a *vax2* (ventral retina)/ *tbx5a* (dorsal retina) *in situ* hybridization probe cocktail to screen 24 hours postfertilization embryos for mutants with altered expression of one or both of these genes. I screened four or more F3 clutches from 64 F2 families and found three novel mutants with required role in dorsal-ventral retinal

patterning. In Chapter 3 I describe our *in situ* hybridization screen, as well as the characterization, mapping, and cloning of one of these mutants, named *bigtop* (*btp*^{zc52}), due to the expanded dorsal expression of *tbx5a* and reduction of *vax2* in the ventral retina.

Characterization of the *btp* mutation revealed expansion of all dorsal markers tested (*tbx5a*, *tbx2a*, *tbx4*, *ephrinB2a*, *gdf6a*, *bmp4*, and *bmp2b*), including especially robust expansion of the *bmp* genes *bmp4* and *bmp2b*. This mutation also results in delayed retinal development after 24 hpf, and altered retinotectal projections. We mapped this mutation to a 4 Mb region on chromosome 2 through mapping services provided by Floragenex, Eugene OR. We then used RNA sequencing to analyze the sequences of mRNAs from pooled siblings and mutants, compared to genomic reference sequence. Using this approach, I hoped to find a nonsense mutation within the 4 Mb mapped region identified by Floragenex; however, I have not yet found such a mutation. The receptor tyrosine kinase axon guidance receptor gene e*phB1* is located within the 4 Mb region identified by Floragenex, and is an excellent candidate for our mutation. Further experiments to analyze the phenotypes of this mutant and test whether *btp* is a mutation in the *ephB1* gene are in progress.

Chapter 4

The final chapter of my dissertation provides a conclusion and discussion of the results of my thesis experiments, and suggests avenues for future research to expand on our results and further clarify the mechanisms of dorsal-ventral retinal patterning and topographic axon projections.

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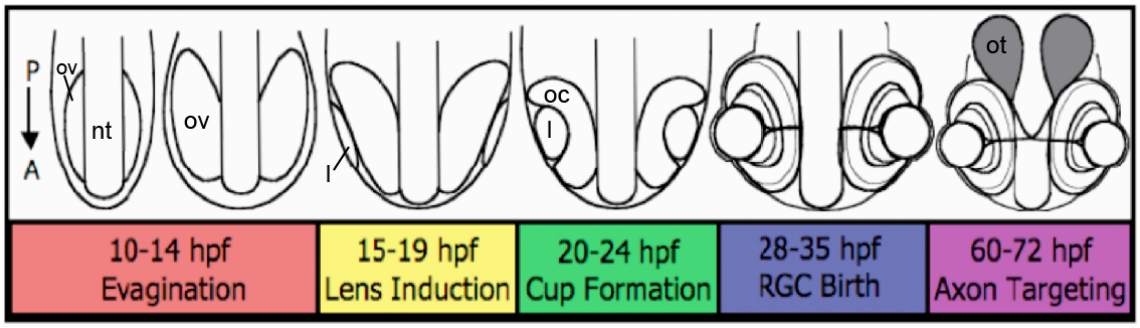
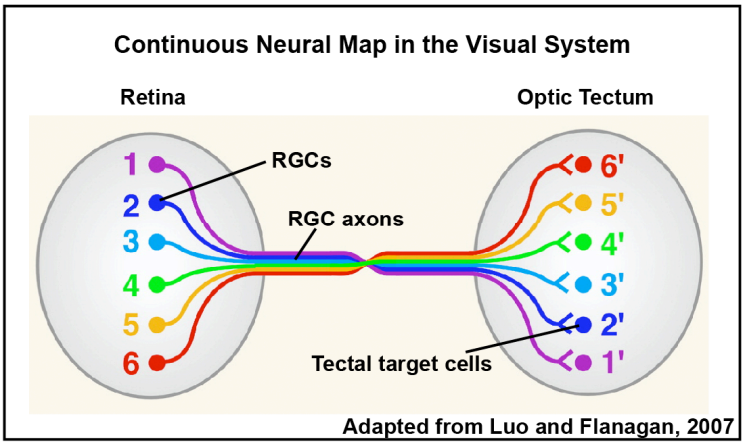


Figure 1: Timeline of retinal development and topographic axon targeting in the zebrafish, shown in a dorsal view. The optic vesicle begins to evaginate from the neural tube at 10 hpf, and continues to enlarge and constrict in the posterior region through 14 hpf. The lens is induced from overlying ectoderm around 15 hpf, enlarging and eventually becoming distinct from the ectoderm. The neural retina undergoes invagination, shaping around the developing lens, and the optic stalk restricts ventrally, forming the optic cup between 20-24 hpf. At 28 hpf, the first retinal ganglion cells (RGCs) are born and begin sending their axons out of the eye. Axon targeting occurs in the brain over a long period, with rough axon pathfinding and targeting complete by 3 dpf. nt, neural tube; ov, optic vesicle; l, lens; oc, optic cup; ot, optic tectum; hfp, hours postfertilization; dpf, days postfertilization.

A



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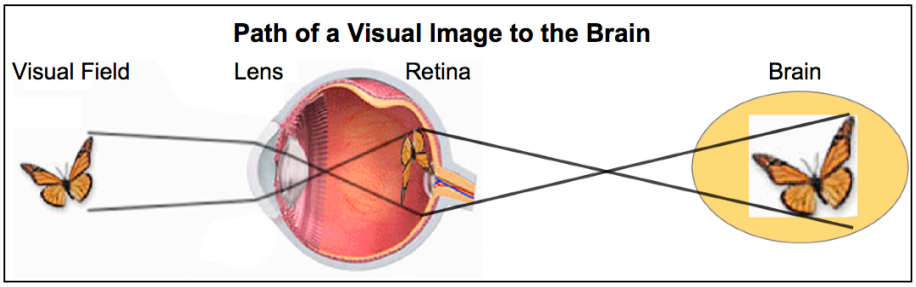

Figure 2: Retinal projections develop in a stereotyped fashion, where neighboring cells of the retina project their axons to neighboring regions of the visual processing centers in the brain. These accurate neural projections allow us to see a spatially organized view of the visual world. We call the precise mapping of retinal ganglion cell axons with their brain targets the retinotopic map. (A) The retinotopic map is an example of a continuous neural map. Within this map, neighboring retinal ganglion cells map to neighboring target cells in the optic tectum. (B) Light enters the eye from the visual world, and is inverted by the lens onto the back of the eye, the neural retina. The neural retina converts the visual light photon signal into neural impulses that are sent to the brain. Axons carrying this neural signal to the brain cross in their path to invert the image seen by the retina again in the brain. Adapted resources.teachnet.ie/torourke/Physicswebsite/Optics.htm.

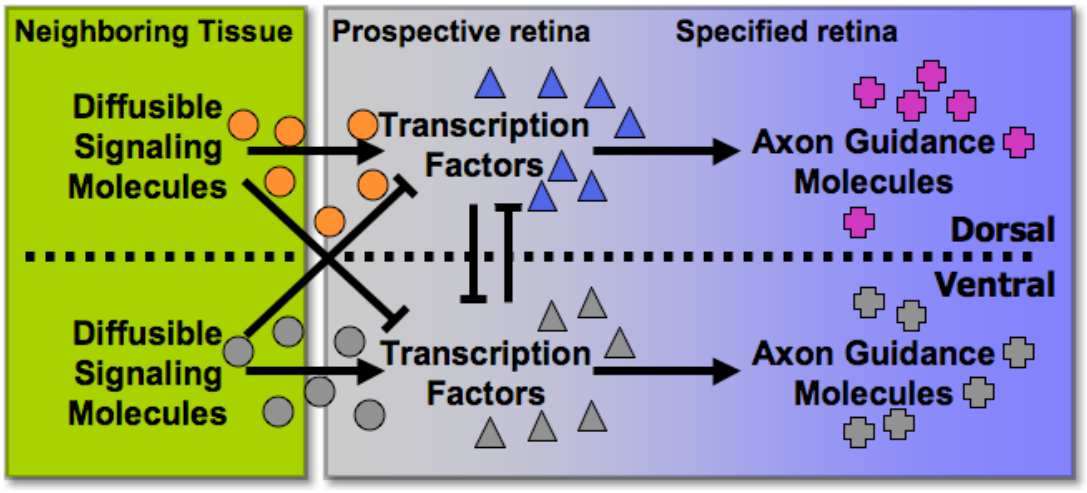

Figure 3: Model for patterning the Dorsal-Ventral axis of the retina, focusing on the dorsal half of this axis. We propose that the dorsal retina could be initiated by a neighboring tissue by upregulation of a signaling molecule, which diffuses into the prospective dorsal retinal region, upregulating dorsal-specific transcription factors in a polarized domain, and downregulating the expression of transcription factors from the prospective ventral retina. Overlapping domains of transcription factors in turn regulate the expression of dorsal specific axon guidance molecules, to form a protein gradient with high levels dorsally and low levels ventrally. Gradients of dorsal-high ventral-low guidance receptors allow the axons expressing them to respond to guidance ligands along their path to the brain, and ultimately map to the correct D-V position on the optic tectum, dependent on their graded D-V position in the retina.

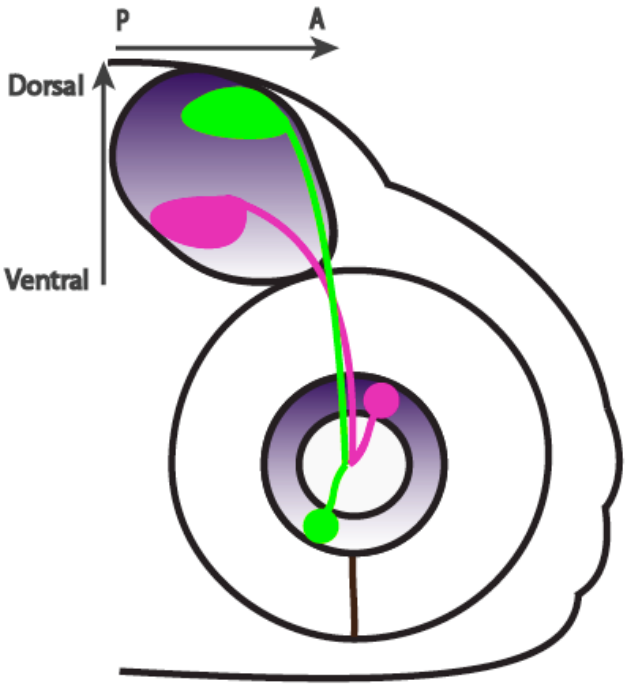

Figure 4: Zebrafish retinotopic mapping. In zebrafish, the primary target of RGCs is the optic tectum. Along the dorsal-ventral axis, dorsal retinal ganglion cells (magenta) map to the contralateral ventral optic tectum, and ventral RGCs (green) map to the contralateral dorsal optic tectum. Anterior-Posterior patterning is also present – anterior retinal cells map more posteriorly on the tectum, and posterior cells map more anteriorly. This topographic map occurs due to molecular patterning of retinal and tectal tissues along dorsal-ventral and anterior-posterior axes. Here a molecular gradient (dark purple) depicts high expression of a patterning molecule dorsal, fading to low expression ventrally.

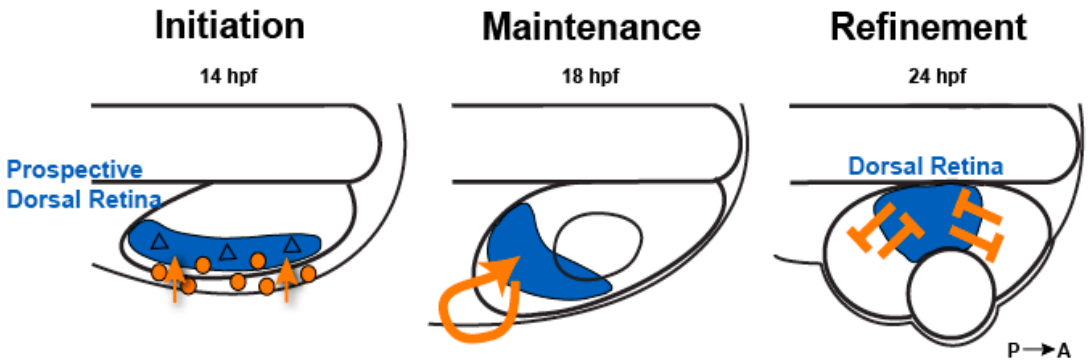

Figure 5: We proposed three phases of dorsal retinal fate specification – initiation, maintenance, and refinement. Figures are dorsal views of the neural tube and a single optic vesicle. Dorsal retinal patterning markers, expressed in polarized patterns within the optic vesicle (blue), are turned on at different times during development. These markers are first observed laterally at 14 hpf, then transition posteriorly in the optic vesicle by 18 hpf, and are located at the dorsal pole (directly opposite the choroid fissure) and centrally behind the lens at 24 hpf. All of these markers maintain their expression over a long time period, and show continued expression at the dorsal retinal pole from 24-72 hpf. We propose that different dorsal fate specification genes (orange) may have specific roles at different stages, with some acting to initiate dorsal polarity, others acting to maintain it, and still others acting to refine the dorsal domain over time.

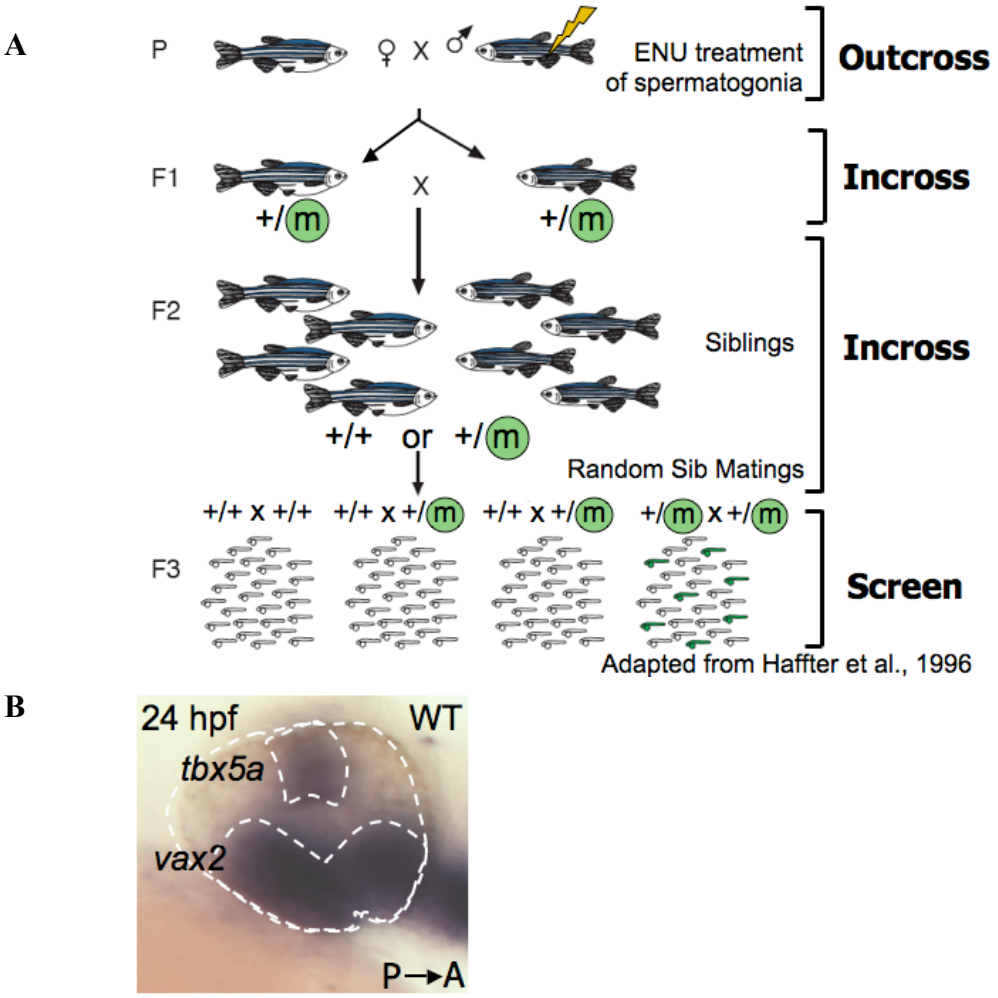

Figure 6: I performed an F3 forward genetic screen to search for D-V patterning mutants. (A) Screen strategy. Male sperm were mutagenized with ethylnitrosurea (ENU), then outcrossed males with wild-type females. F1 offspring were incrossed to double the mutational load, and offspring from these crosses were grown in F2 families, heterozygous for random mutations throughout the genome. Pairs of F2 family siblings were then incrossed to generate homozygous F3 mutants, which would be expected to be ¼ of the offspring in ¼ of the sibling crosses. (B) *tbx5a/vax2 in situ* probe cocktail. I screened F3 clutches at 24 hpf for altered expression of D-V patterning genes

CHAPTER 2

EXTRAOCULAR ECTODERM TRIGGERS DORSAL RETINAL FATE DURING OPTIC VESICLE EVAGINATION IN ZEBRAFISH

Abstract

Dorsal retinal fate is established early in eye development, via expression of spatially restricted dorsal-specific transcription factor genes in the evaginating optic vesicle; yet the events leading to initiation of dorsal fate are not clear. In light of standard tissue patterning models, I hypothesized that dorsal fate induction would require an extraocular signal, arising from a neighboring tissue, to pattern the prospective dorsal retina. However no extraocular initiation signals have been identified. Past research has primarily focused on signaling molecules within the optic vesicle, and the tissue of origin of dorsal initiating signals is currently unknown. I used the zebrafish embryo to determine the source of the dorsal retina-inducing signal.

Extensive cell movements occur during zebrafish optic vesicle morphogenesis, however the location of prospective dorsal cells within the early optic vesicle and their spatial relationship to early dorsal markers is currently unknown. My mRNA expression and fate mapping analyses demonstrate that the dorsolateral optic vesicle is the earliest region to express dorsal specific markers and cells from this domain contribute to the dorsal retinal pole at 24 hpf.

I show that three *bmp* genes, *gdf6a*, *bmp2b*, and *bmp4*, are expressed extraocularly in potential initiation tissues before retinal patterning begins, and these same genes are expressed in polarized patterns dorsally at 25 hpf. I show here that the *gdf6a* dorsal initiation signal arises from the extraocular non-neural ectoderm during optic vesicle evagination. I find that *bmp2b* is involved in dorsal retina initiation, acting upstream of *gdf6a*. Together, this work has identified the nature and source of extraocular signals required to pattern the dorsal retinal axis.

Introduction

Achieving precise retinotopic axon targeting during development requires the patterning of projecting (retina) and target (brain) tissues along anterior-posterior and dorsal-ventral axes, such that individual cells of both tissues acquire a molecularly specified positional identity (McLaughlin et al., 2003; McLaughlin and O'Leary, 2005). Regarding the first step in fate specification, I hypothesized that the dorsal retinal initiating signal would be a diffusible molecule, providing asymmetric positional information to prospective dorsal retina, from a location external to the eye field. However an extraocular initiator of dorsal retinal fate has not yet been identified.

Past research has identified several genes necessary for establishing dorsal retinal fate (McLaughlin et al., 2003; Murali et al., 2005; Behesti et al., 2006; Sakuta et al., 2006; Asai-Coakwell et al., 2007; French et al., 2007; Plas et al., 2008; French et al., 2009; Gosse and Baier, 2009) but has focused on expression and regulation within the retinal field. Historically, a simple model was set forth in which dorsal expression of *bmp4* initiates dorsal *tbx5* and downregulates ventral *vax2* (McLaughlin et al., 2003). In support of this model, *Bmp4* knockout mice never initiate expression of *Tbx5a* (Murali et

al., 2005). Bmp4 gain of function experiments, and loss of function of receptors, also suggest Bmp-mediated regulation of *Tbx5* in the dorsal retina (Murali et al., 2005; Behesti et al., 2006). However extraocular factors for initiating *bmp4* expression are unknown, and *Bmp4* null mice die shortly after E9.5, precluding further analysis of gene expression and retinotectal topography in this model (Murali et al., 2005). Furthermore, recent work in zebrafish suggested that *bmp4* does not initiate dorsal-specific gene expression, whereas the *Bmp* family gene *gdf6a* plays a critical role (French et al., 2009). Gdf6a is a Bmp family member known to affect eye development in humans and mouse (Asai-Coakwell et al., 2007; Asai-Coakwell et al., 2009), yet its role in dorsal retinal patterning in these species has not yet been analyzed. In zebrafish, *gdf6a* is expressed extraocularly during optic vesicle evagination, and in the prospective dorsal retina from 15-48 hpf. *gdf6a* is critical for dorsal initiation, yet it has not been determined what time, and from what tissue, *gdf6a* acts. It is unknown whether *gdf6a* is an extraocular initiator of dorsal retinal fate, or acts only within the retinal field, suggesting another extraocular signal necessary for initiating *gdf6a* within the retina. I set out to determine the identity, time of action, and tissue source of an extraocular initiator, as well as the domain of the optic vesicle first initiated with dorsal-specific markers.

The first known manifestation of retinal dorsal identity in zebrafish is the expression of the T-box transcription factor genes, *tbx2a* and *tbx5a*, in restricted optic vesicle domains (Veien et al., 2008). The primary candidates for regulation of *tbx* genes, and thus initiation of dorsal identity, are the morphogens of the bone morphogenetic protein family (Bmps). Five *bmp* genes mark the dorsal domain of the zebrafish eye at 24 hpf—*bmp2a, bmp2b*, *bmp4*, *bmp7b* and *gdf6a* (Thisse, 2001; Thisse, 2005; Shawi and

Serluca, 2008). The continuing expression of all known retinal patterning genes from early to late stages of eye development led us hypothesize that these *bmps* were likely candidates for dorsal initiation signals. However it is unknown which of these candidate morphogens are expressed early, during optic vesicle evagination, in extraocular spatiotemporal domains where they could initiate the prospective dorsal retina transcription factors. Furthermore, it is not known which region of the early optic vesicle is poised to receive the dorsal initiation signal. Recent research analyzed the 24 hpf fates of early optic vesicle cells (Kwan et al., 2012), and found extensive cell movements during this time period. I theorized that extraocular dorsal initiation signals should be located adjacent to the region of the earliest polarized expression of dorsal fate within the retina. Tissues adjacent to the early optic vesicle that could give rise to the initiation signal include the neural tube (medial), non-neural ectoderm (dorsal and lateral), and prechordal plate mesoderm (anterior). In this study, I show that the lateral region of the early optic vesicle, which first expresses dorsal specific transcription factors, and is adjacent to non-neural ectoderm, is fated to give rise to the dorsal retina.

I analyzed the precise timing of dorsal initiation using a pharmacological inhibitor of Bmp signaling, and found that Bmps are required for initiating dorsal retinal markers prior to their expression within the optic vesicle, indicating that bmps are initiating dorsal retina by signaling from an extraocular tissue. I analyzed the spatiotemporal domains of genes encoding candidate initiators–*gdf6a*, *bmp2b*, and *bmp4*, and using mutants I determined the necessity of these genes for initiating dorsal fate in zebrafish.

My work shows that dorsal retinal fate is initiated by extraocular *gdf6a*, arising from the non-neural head ectoderm at 11.5 hpf. Adjacent tissue of the dorsolateral optic

vesicle leaflet receives this signal and upregulates T-box transcription factors in this domain. Additionally, I find that *bmp2b* is necessary for dorsal retinal initiation, acting upstream of *gdf6a*, possibly to establish its expression in the extraocular ectoderm.

Methods

Animals

I maintained adult zebrafish (*Danio rerio*) on a 14-hour light, 10-hour dark cycle. I raised embryos in E3 embryo medium with methylene blue at 28.5 °C, anesthetized with 0.2mg/ml tricaine and fixed in 4 % paraformaldehyde unless otherwise noted. I staged embryos according to Kimmel et al., (Kimmel et al., 1995), by counting somites (morphologically) and/or by *in situ* using the somite marker *myoD*. I used Tübingen strain embryos for Kaede fate map and Bmp inhibitor (LDN 193189) experiments.

Mutants

I used four mutant lines for this work: *bmp2b* ^{tc300} (Mullins et al., 1996), *bmp4* ^{st72} (Stickney et al., 2007), *gdf6a* ^{s327} (Muto et al., 2005) and *oep* ^{m134} (Schier et al., 1997; Zhang et al., 1998). The *bmp2b* ^{tc300} line was generated in the Tübingen strain background; *bmp4* ^{st72}, and *gdf6a* ^{s327} were generated in Tupfel long fin (TL) wild type strain and the *oep* ^{m134} line was generated in the AB wild type strain. I maintained all mutant lines on their original backgrounds.

Genotyping

I genotyped adult fin clips and whole mount individual *in situ* embryos using Derived Cleaved Amplified Polymorphic Sequences (dCAPS) PCR. PCR primers for

gdf6a ^{s327} and *bmp4* ^{st72} were previously described by (Stickney et al., 2007; Gosse and Baier, 2009) respectively. I generated additional primers for genotyping *bmp4* ^{st72} and *bmp2b* ^{tc300} using the web-based program dCAPS Finder 2.0 (Neff et al., 2002). *bmp4* ^{st72} Fwd: TGGTGAGGCACAACACCTCCAACTAG, Rev: CCGAGTCAGCGGGTGACTTTTGCCGTC. SpeI cuts mutant band. *bmp2b* ^{tc300} Fwd: GAAGTATCCGAGGAGGCTGA Rev: CCTCCACCACCATGTCCT. HaeIII cuts mutant band.

In situ hybridization

I performed whole mount *in situ* hybridization to analyze mRNA expression as described by Thisse and Thisse, (Thisse and Thisse, 2008) with the following modifications: I incubated and washed some of the samples using a Biolane HTI *in situ* machine (Huller and Huttner AG, Tübingen, Germany). I synthesized labeled riboprobes (fluorescein-UTP-labeled for *rx3*, all others labeled with Digoxigenin-UTP) using in vitro transcription RNA labeling kits from Roche. Probes were as follows: *tbx2a* (Dheen et al., 1999), *tbx5a* (Ruvinsky et al., 2000), *bmp4* (gift, M. Mullins, University of Pennsylvania), *tbx4*, *bmp2b* (Nikaido et al., 1997), *vax2* (Take-uchi et al., 2003), *pax2a*, *rx3*, *gdf6a* (Veien et al., 2008), *pitx3*, *ephrinB2a* (Durbin et al., 1998), *myoD* (Weinberg et al., 1996) and *isl1* (Inoue et al., 1994). Digoxigenin probes were developed with BM-Purple, and the fluorescein *rx3* probe was developed using INT-BCIP (Roche Applied Science). I cleared whole mount embryos in 50-80% glycerol and imaged them using an Olympus SZX 12 stereomicroscope, an Olympus SN1H045411-H camera, and Picture Frame™ imaging software version 2.3. Sectioned embryos were embedded in plastic according to (Sullivan-Brown et al., 2011), and sectioned transversely at either five or 12

μm thickness using a Reichert-Jung 2050 microtome. All sections were imaged on an Olympus BX51WI inverted microscope using the same camera and imaging software as for whole mount images. In cases where a particular mutant phenotype could not be identified, I imaged and genotyped individual embryos to confirm that the pictures presented contained the correct genotypes.

Kaede Fate Map

I prepared capped *NLS-Kaede* mRNA using the mMessage mMachine kit from Ambion. I injected 2 ng (500 ng/μL) *NLS-Kaede* mRNA into 1-cell stage zebrafish embryos according to (Hatta et al., 2006), and raised them in E2 with gentamycin (E2/GN). At 11 hpf I dechorionated and mounted embryos live in 1.5 % low melting temperature agarose to image them from the dorsal side on an FV1000-XY Olympus IX81 confocal microscope. Images shown are maximum intensity projections rendered using ImageJ.

Bmp inhibitor LDN 193189

LDN 193189 (Stemgent) was dissolved in 100% DMSO. I incubated dechorionated embryos in 1 μM LDN 193189, 1% DMSO in E2/GN or equal volume 1% DMSO/E2/GN as a control. I began the treatments at 4 or 9.5 hpf, and fixed at 13 hpf. I assayed treated embryos using *in situ* hybridization for expression of the early eye field marker *rx3* or the dorsal-specific transcription factor *tbx5a*.

Results

tbx5a expression is broad and dynamic during eye morphogenesis

tbx5a expression is thought to mark prospective dorsal fate in the early optic vesicle. Because expression of this marker begins at 12 hpf (Veien et al., 2008), and is one of the earliest markers of dorsal fate, I hypothesized that the early expression domain of *tbx5a* may indicate the likely extraocular location of dorsal initiation signals. I therefore analyzed changes in the expression domain of *tbx5a* mRNA in the optic vesicle following its onset at 12 hpf, over time until 24 hpf. *tbx5a* expression is initiated in the lateral half of the dorsal optic vesicle leaflet at 12 hpf (Figure 7, A0-A3). The early dorsolateral expression domain begins to shift posteriorly and medially, and at 14 hpf occupies both the dorsal and ventral optic vesicle leaflets in the posterior optic vesicle, while remaining dorsolateral in the anterior domain (Figure 7 B0-B3; compare arrowheads in B3 and A3). *tbx5a* expression continues to move posteriorly over time (C0, D0), primarily occupying the prospective retinal (dorsal) leaflet (arrowhead in C3), with the posterior portion of expression in a more medial domain, and the anterior portion more lateral to create a teardrop shape in the whole mount dorsal view between 15-18 hpf (Figure 7 C0-C3 and D0-D3). At 24 hpf *tbx5a* expression is opposite to the choroid fissure in the dorsal retina, and also extends into the central retinal domain behind the lens (Figure 7 E0-E3). The dynamic expression of *tbx5a* suggests that early expression of this marker may mark the process of dorsal induction. Additionally, the early expression domain in the dorsolateral optic vesicle suggests an initiating signal could arise from the overlying extraocular ectoderm (arrow in Figure 7 A1).

Movements of prospective dorsal cells resemble *tbx5a* mRNA expression domain changes over time

Tissue patterning, via combinatorial expression and interactions of transcription factors, can commit specific cells to a particular cell fate early during development. According to this model, I hypothesized that a portion of the cells of the dorsolateral optic vesicle expressing the dorsal marker *tbx5a* at 12 hpf would undergo morphogenesis to reside in the dorsal retina at 24 hpf; To test this, I used the photoconvertible fluorescent protein Kaede to fate map a portion of the *tbx5a* 12 hpf expression domain from 14-22 hpf (Figure 8). I photoactivated the lateral optic vesicle of live 14 hpf embryos ubiquitously expressing Kaede (Figure 8 A, B), and took sequential images to determine the fates of these lateral cells over time (Figure 8 C-D). In accordance with my hypothesis, cells within the Kaede activated lateral optic vesicle domain show a pattern of movement over time similar to the dynamic *tbx5a* mRNA domain (Figure 8 B'-D'). First, photoactivated lateral cells (magenta, Figure 8 B) move to the posterior optic vesicle by 18 hpf (Figure 8 C-C'), then populate the dorsal pole and central retinal domains extending from dorsal to behind the lens more ventrally at 24 hpf (Figure 8 D-D'). This final domain is triangular in shape, extending from the widest point dorsally, to a triangular tip more centrally and ventrally, very similar to the 24 hpf domain of *tbx5a* (Figure 7 E0-E3). These results indicate that dorsal retinal fate may be fixed over time—a portion of cells specified as dorsal and expressing *tbx5a* at 12 hpf populate the dorsal pole of the retina and maintain their expression of *tbx5a* at 24 hpf.

Dorsal-initiating Bmp signals are acting prior to expression of *bmps* within the retina

Previous work shows that *bmps* are necessary for initiating the expression of several dorsal markers, (Murali et al., 2005; French et al., 2009) however it is not known at what time these signals are acting. I performed pharmacological experiments using a selective Bmp inhibitor to analyze the time window in which Bmps are required for initiating dorsal marker expression. LDN193189 blocks phosporylation of Smad 1/5/8 proteins by type 1 Bmp receptors, and is therefore expected to block all *bmp* signaling. I specifically tested whether Bmps are required for dorsal retinal initiation prior to their expression in the retina. Treatment with 1 μM LDN from 4-13 hours leads to strongly dorsalized phenotypes (Cannon et al., 2010). I found that these dorsalized embryos do form an eye field when assayed with the *rx3* eye field marker *in situ* probe (Figure 9 A-B). However these embryos fail to initiate the dorsal marker *tbx5a* (Figure 9 C-D). When I treated with 1 μM LDN beginning at 9.5 hpf, again fixing at 13 hpf, normal eye fields are formed as in the DMSO control (Figure 9 E-F), yet dorsal *tbx5a* is not expressed (Figure 9 G-H). These results show that Bmps are acting between 9.5 and 13 hpf to initiate dorsal retinal fate. Importantly, this is before expression of these genes is activated in the retina (Veien et al., 2008), indicating that Bmps are acting as extraocular initiators of dorsal fate.

Both *bmp2b* and *gdf6a* are necessary for initiation of dorsal retinal fate

Bmps are diffusible signaling molecules that can act in a gradient far from their source, and previous reports in several species implicate Bmps in dorsal fate initiation

(Murali et al., 2005; Behesti et al., 2006; Plas et al., 2008; French et al., 2009). In zebrafish, several members of the *bmp* family are expressed surrounding the prospective dorsal retina, and therefore are likely candidates as extraocular initiators of dorsal fate. I first examined the 11-13 hpf expression patterns of *bmp* genes with known expression dorsally in the retina at 24 hpf. To determine whether these *bmps* might encode extraocular initiators of dorsal fate, I next examined expression of dorsal fate markers shortly after initiation in null mutants for each of these genes, allowing us to determine the complete loss of function phenotype.

The *bmp4* gene is expressed anteriorly to the evaginating optic vesicle at 12-14 hpf, in the prechordal plate (Figure 10 A). However, I found that the prechordal plate is not necessary for dorsal retina initiation. The zebrafish mutant *one-eyed pinhead* (*oep* ^{m134}) does not form prechordal plate tissues, as evidenced by loss of *bmp4* and *isl1* expression in this region (Figure 10 A-D, arrows indicating prechordal plate in C and absent tissue in D). Yet the dorsal marker *tbx5a* is initiated normally in *oep* mutants (Figure 10 E-F), suggesting that *bmp4* from this tissue does not participate in dorsal retinal fate specification. Furthermore, in agreement with previous morpholino studies (French et al., 2009) and contrary to results in mouse (Murali et al., 2005), the polarized dorsal-ventral markers *tbx5a*, *gdf6a*, *ephrinB2a*, and *vax2* showed normal expression in *bmp4* ^{st72} null mutants (Figure 11 A-H).

The *bmp* family member *gdf6a* is expressed in the extraocular ectoderm between 11-15 hpf and in the prospective dorsal retina at 15-24 hpf (Thisse, 2001). Dorsal axons project to the ventral optic tectum in WT, but they mistarget dorsally in *gdf6a* ^{s327} null mutants, leaving the ventral optic tectum devoid of axons in this mutant (Gosse and

Baier, 2009). Morpholino knockdown experiments have shown that *gdf6a* is important for establishing dorsal-ventral patterning within the retina, as assayed by expression domains of *tbx5a*, *bmp4*, *bambi* and *vax2* (French et al., 2007); yet the required time and location of this signal have not been determined. I found that the dorsal markers *tbx5*, *bmp4* and *bmp2b* are never initiated in *gdf6a* mutants, and the dorsal markers *tbx2a* and *tbx4* are initiated in smaller domains and at lower intensity than in siblings (Figure 12 A-N). At later time points (18-26 hpf), the dorsal markers *tbx2a*, *tbx5*, *tbx4*, *bmp2b*, *bmp4*, *ephrinB2a*, *ephrinB1* and *raldh2* are completely absent in *gdf6a* mutants [Figure 13 A-P; see also (Gosse and Baier, 2009)]. Furthermore, markers of ventral retinal fate including *vax2* and *ephB2* are initiated normally in *gdf6a* mutants, then expand following their initiation (*vax2*, Figure 12 O-P), and are expressed throughout most of the optic vesicle at 18-26 hpf (*vax2*; *ephB2,* Figure 13 Q-T). These data show that dorsal retinal fate is never properly initiated in *gdf6a* mutants, and confirm previous work showing that *gdf6a* is a critical initiator of dorsal retinal fate.

bmp2b is expressed surrounding the evaginating optic vesicle at 11-15 hpf in WT (Figure 14 A), and this expression domain is unaffected in *gdf6a* mutants (Figure 14 B). A null mutation in the *bmp2b* gene (*bmp2b* ^{tc300}) results in severe developmental defects, and an early death at 14-16 hpf (Mullins et al., 1996). I was able, however, to examine the earliest stages of dorsal eye specification in this mutant. First, expression of *rx3*, an early eye field marker, in *bmp2b* mutants and siblings showed that *bmp2b* mutants establish an eye field between 12-14 hpf (Figure 14 C-F). I next analyzed the expression of *tbx2a* at 12 hpf and *tbx5a* at 13 hpf, and found that *tbx2a* was initiated in a much smaller domain compared to wild type (Figure 14 G-H), and *tbx5a* was never initiated

(Figure 14 I-J). Furthermore, the earliest marker of the prospective ventral optic stalk domain, *pax2a*, is normally initiated in *bmp2b* mutants (Figure 14 K-L, arrows mark retinal staining, arrowheads point to midbrain-hindbrain boundary). These results show that dorsal retinal markers are not properly initiated in *bmp2b* mutants, identifying a novel role for *bmp2b* in dorsal fate specification.

It is important to note that retinal expression of *bmp2b* is never present in *gdf6a* mutants (Figure 12 M, N; Figure 13 G, H), suggesting that *gdf6a* acts upstream of *bmp2b* within the retinal field. However, my results showing that early extraocular *bmp2b* expression is normal in *gdf6a* mutants (Figure 14, A-B), suggest that the extraocular *bmp2b* necessary for dorsal initiation is acting upstream of *gdf6a*.

<u>The dorsal initiation signal arises from the extraocular ectoderm adjacent to the dorsolateral optic vesicle at 11-12 hpf</u>

Loss of the *gdf6a* initiation signal leads to near-complete loss of dorsally expressed genes and expansion of ventral fate toward the dorsal pole, but it was not clear in which tissue *gdf6a* is acting. To examine the precise location of *gdf6a* expression, I performed double *in situ* hybridization with *gdf6a* and *rx3* probes developed in two colors, and imaged optic vesicles both in whole mount views and in tissue sections (Figure 15 A-J, *gdf6a* – blue, *rx3* - orange). *gdf6a* mRNA is expressed in the extraocular head ectoderm beginning at 11 hpf, immediately prior to and directly adjacent to the region of the lateral optic vesicle that expresses *tbx5a* (Figure 15 A, C, E, I, ; compare to Figure 7 A0-A3). The extraocular *gdf6a* expression pattern is highly dynamic between 11 and 13 hpf. At the 11 hpf time point, the strongest *gdf6a* expression is posterior and anterior to the optic vesicle, with only faint staining laterally (Figure 15 A, arrows for

strong anterior and posterior staining, arrowhead for faint lateral staining). By 11.5 hpf, the lateral domain of *gdf6a* expression has intensified (arrowhead in Figure 15 C), and begins to expand over the dorsolateral half of the optic vesicle (Figure 15 C, arrow in I). This expansion and intensification continues, and at 12-13 hpf, *gdf6a* expression in the extraocular ectoderm covers the dorsal leaflet of the optic vesicle (Figure 15 E, G, J). The location and time of *gdf6a* expression extraocularly suggests that it is the final critical initiator of *tbx2a* and *tbx5a* expression in the cells of the dorsolateral optic vesicle (Figure 7 A0-A3), specifying dorsal fate within the retina.

Interestingly, *bmp2b* mutants have been shown to lack non-neural ectoderm (Nguyen et al., 1998), and therefore would not be expected to initiate *gdf6a* expression extraocularly, as this signal arises from the non-neural lateral head ectoderm. I therefore analyzed extraocular *gdf6a* expression in *bmp2b* mutants from 11-13 hpf. Indeed, I found that *bmp2b* mutants have increased anterior *gdf6a* expression (Figure 15 D, F arrows), but completely lack the *gdf6a* expression surrounding the lateral and dorsal optic vesicle (Figure 15 B, D, F, H arrowheads). Similarly, the lens placode marker *pitx3* is also absent in *bmp2b* mutants (Figure 15 K-M, arrows in K-L). These results are consistent with the lack of initiation of dorsal retinal fate in *bmp2b* mutants, due to a lack of a dorsal initiation signal from lateral non-neural ectoderm, again showing that extraocular *bmp2b* acts upstream of extraocular *gdf6a* in dorsal retina specification.

Discussion

Topographic connections of retinal axons with their brain targets allow us to perceive a spatially organized image of the visual world. Choreographing accurate axon targeting during development first requires the molecular patterning of retinal and tectal

(brain) tissues along anterior-posterior and dorsal-ventral axes. Axial patterning often begins with a morphogen gradient that arises from a neighboring tissue, leading to initiation of transcription factors within the tissue of interest, and hence a graded fate along the axis. My data demonstrate that dorsal retinal patterning employs such a mechanism (Figure 16). Initiation of dorsal retinal fate first requires expression of *bmp2b*. Previous research (Nguyen et al., 1998) and my experiments both indicate that *bmp2b* mutants never form non-neural ectoderm, the critical tissue for dorsal fate initiation by *gdf6a*. Non-neural ectoderm, itself expressing *bmp2b* as well as *gdf6a*, surrounds the evaginating optic vesicle between 10 and 15 hpf. *gdf6a* is likely the critical initiator of dorsal fate within the retina (French et al., 2009), and without this signal *tbx5a* and *tbx2a* fail to be initiated within the dorsolateral leaflet of the optic vesicle at 12 hpf. My data show that the *gdf6a* initiation signal acts between 11-12 hpf, a time when this gene is not expressed within the retinal field but is expressed in the surrounding ectoderm. Pharmacological manipulations using the selective Bmp inhibitor LDN corroborate these results, showing that Bmps are necessary for dorsal initiation after 9.5 hpf.

Polarized expression of the prospective dorsal marker *tbx5a* is broad and dynamic during retinal morphogenesis. Initially, *tbx5a* marks the lateral half of the dorsal optic vesicle leaflet after dorsal initiation. Subsequently these cells undergo dramatic movements during morphogenesis of the optic cup. Kaede fate mapping shows that the dynamic pattern of *tbx5a* expression generally recapitulates the movements of these cells. However, not all cells expressing this gene reside in the dorsal pole at 24 hpf. Thus, my data indicate that cells of the lateral optic vesicle at 12 hpf contribute to both the dorsal and central retinal domains at 24 hpf. Consistent with these findings, four dimensional

cell tracking (Kwan et al., 2012) indicates that cells of the 12 hpf posterior-lateral dorsal optic vesicle leaflet give rise to dorsal pole cells at 24 hpf, and the central-lateral dorsal optic vesicle leaflet at 12 hpf gives rise to cells behind the lens at 24 hpf. The expression domain of *tbx5a* is reduced to only a small triangle at the dorsal pole by 32 hpf, lending support to the idea that some cells eventually turn off this marker. Therefore, while early *tbx5a* expression is a marker of the potential for dorsal fate, many cells initially expressing this gene may ultimately reside in other regions.

While I believe that the primary role of *bmp2b* in dorsal fate initiation is in formation of non-neural ectoderm, I cannot rule out a later secondary role for this gene in initiation during optic vesicle evagination. *bmp2b* mRNA is expressed extraocularly in the lens placode at 11-13 hpf (Figure 14 A), overlapping with *gdf6a* expression (Figure 15 G), and could cooperate with *gdf6a* to initiate dorsal fate during evagination. Similarly, *bmp4* is expressed within the retinal field beginning at 14 hpf (Veien et al., 2008), and could be interacting with *gdf6a* at this later time point. Dorsal character is not entirely absent in *gdf6a* mutants (Figure 12 D, L arrowheads, Figure 13 L, T arrowheads), suggesting the possibility that multiple Bmps could cooperate to initiate dorsal fate during optic vesicle evagination. However my analysis of different allelic combinations of *gdf6a*, *bmp2b*, and *bmp4* null mutations (not shown), was unable to conclusively demonstrate genetic interactions that would support combinatorial signaling mechanisms.

Despite the critical role of *gdf6a* for dorsal retina initiation in zebrafish, a similar role for this factor in other species has not been described. In other vertebrates, *Gdf6* is expressed in the dorsal retina and its loss results in a variety of ocular malformations including colobomata, microphthalmia and anopthalmia (Hanel and Hensey, 2006; Asai-

Coakwell et al., 2007; Asai-Coakwell et al., 2009). These gross anatomical phenotypes may be a result of defective dorsal-ventral patterning in these species as well. Additionally, while expression of *Bmp2* in dorsal retina is conserved across species, the function of this gene in dorsal fate specification is also incompletely understood. Intriguingly, *Bmp2* plays a critical role in maintenance of dorsal fate in chick, but has no apparent role in dorsal initiation (Sakuta et al., 2006). Characterization of later roles for *bmp2b* in dorsal retinal maintenance in zebrafish will require conditional inactivation, as global mutants die at 14-16 hpf. Interestingly, contrary to the observations in mouse (Murali et al., 2005), where *bmp4* plays a critical role in initiation of dorsal fate, I have not found any role for this gene in zebrafish retinal patterning. It is possible that different *Bmp* genes have exchanged functions in eye development through evolution, as has been shown previously during gastrulation (Sasai, 2001).

In summary, my study has identified the extraocular location and time of action of the dorsal retinal initiation signal. I have also demonstrated a novel role for *bmp2b* in dorsal initiation, acting in the formation of non-neural ectoderm, the critical source of the initiation signal. My work defines a basic mechanism for retinal patterning using extraocular tissues, and while the precise location and identity of this signal may differ between vertebrate species, it is likely that the overall strategy is conserved.

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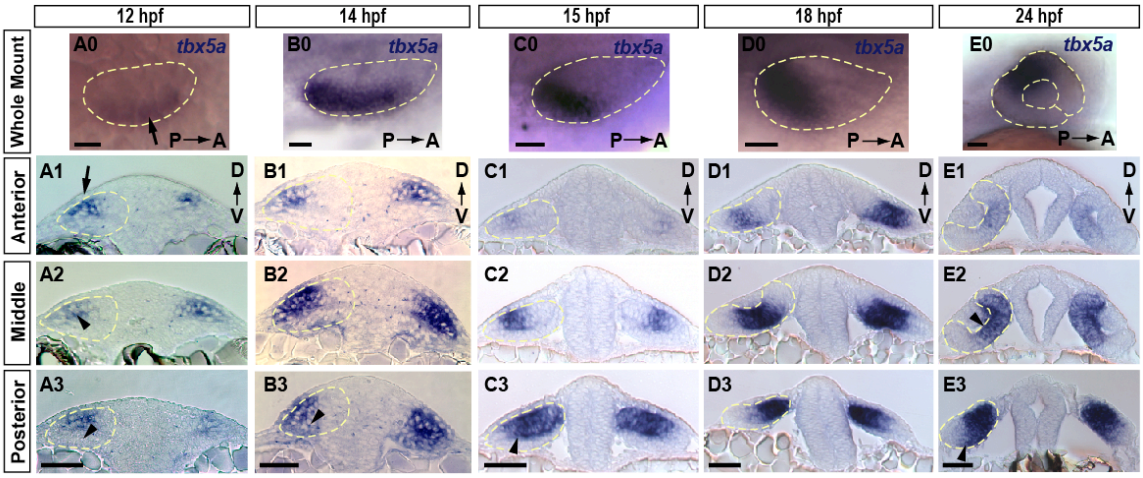

Figure 7. The expression domain of prospective dorsal marker *tbx5a* is broad and dynamic. Expression of *tbx5a* in the developing retina at (A) 12, (B) 14, (C) 15, (D) 18 and (E) 24 hpf (blue). 0 wholemount; 1-3, transverse sections. (A) *tbx5a* mRNA is expressed in dorsolateral optic vesicle (arrow in A0, arrowhead in A2) at 12 hpf, and is absent from the ventral leaflet (arrowhead in A3). Extraocular ectoderm is directly overlying this portion of the optic vesicle (arrow in A1). (B) At 14 hpf expression extends to the ventral leaflet in posterior sections (arrowhead in B3). (A0, B0 dorsal views). (C-D) *tbx5a* domain moves posteriorly from 15-18 hpf and resides primarily in the dorsal (prospective retinal) leaflet (arrowhead in C3). (C0, D0, dorsal views). (E) *tbx5a* expression still covers much of the eye at 24 hpf, including prospective dorsal pole, central retinal domain behind lens (arrowhead in E2), and extending to the ventral domain (arrowhead in E3) (E0, lateral view). Scale Bars = 50 µm. Dashed yellow lines outline optic vesicles in all images, also lens and choroid fissure in E0.

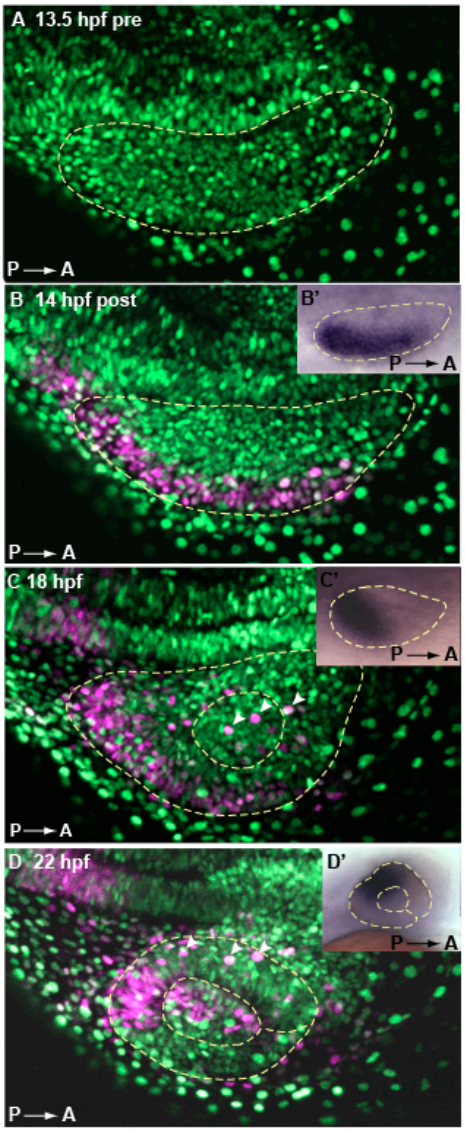

Figure 8. Cells of the lateral optic vesicle give rise to prospective dorsal and central retina. A Kaede fate map of 14 hpf lateral retina shows that cell movements generally recapitulate movements of *tbx5a* expression, indicating that dorsal fate initiation could be a discrete early event. Dashed yellow lines outline optic vesicles (A-D'), lenses (C-D'), and choroid fissure (D-D'). (A) Preactivation, ubiquitous NLS green Kaede, 13.5 hpf. (B) 14 hpf, immediately after photoactivation, cells of lateral optic vesicle express photoactivated Kaede (magenta). (B') 14 hpf whole mount *tbx5a* expression, dorsal view (C) 18 hpf, photoactivated Kaede cells from lateral optic vesicle have moved posteriorly. (C') 18 hpf whole mount *tbx5a* expression, dorsal view (D) 22 hpf, photoactivated magenta cells reside primarily in the dorsal and central retinal domains, as well as in the lens. (D') 24 hpf whole mount *tbx5a* expression, lateral view. Movements of photocleaved Kaede 14 hpf lateral retinal cells are generally consistent with the pattern of movement of *tbx5a*—compare magenta domains in A-D to A'-D' insets and to Figure 7 sections. White arrowheads in C and D mark extraocular ectodermal cells overlying the retina and lens.

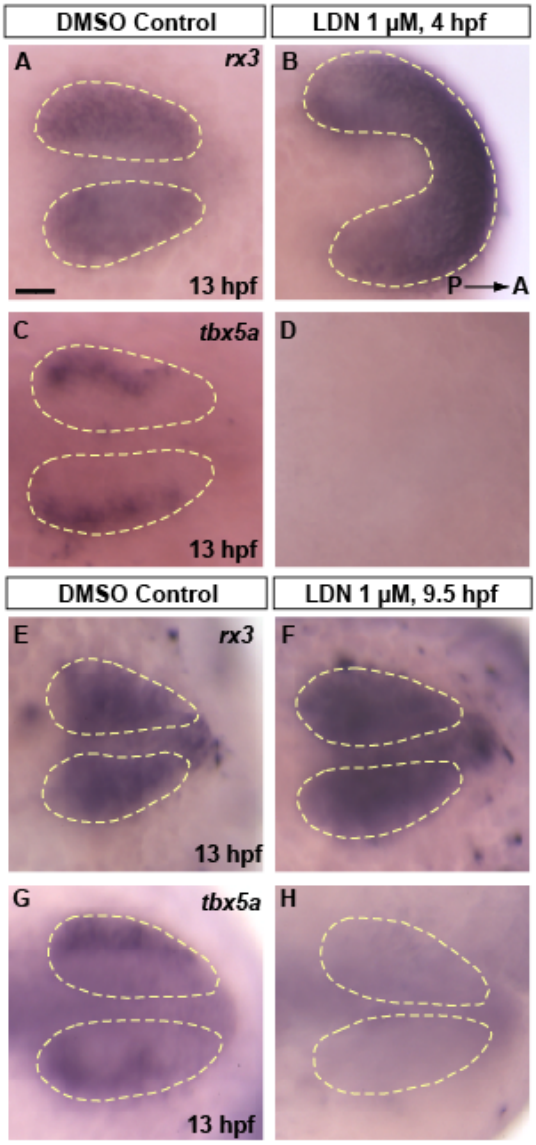

Figure 9. bmp signals necessary for dorsal fate initiation are acting between 9.5 hpf and 13 hpf. Pharmacological inhibition of *bmp* signaling using the small molecule inhibitor LDN 193189 prevents initiation of dorsal fate in the retina. (A, B) *rx3* expression following 4-13 hpf continuous treatment with 1% DMSO in E2/GN (control) or 1 µM LDN 193189 in 1% DMSO E2/GN shows that treatment with LDN 193189 leads to severely dorsalized embryos, yet these embryos still form an eye field. (C, D) Contrary to DMSO controls, eye fields in LDN treated embryos fail to initiate expression of the prospective dorsal marker *tbx5a*. (E, F) Embryos treated from 9.5-13 hpf with 1% DMSO in E2/GN (control) or 1 µM LDN 193189 in 1% DMSO E2/GN both show normal *rx3* expression, indicating normal eye field formation. (G, H) However, contrary to controls, optic vesicles in treated embryos fail to initiate expression of the prospective dorsal marker *tbx5a*, suggesting that dorsal fate is never initiated if *bmp* signaling is inhibited after 9.5 hpf. All images are dorsal views, anterior to the right. Scale bar = 50 µm. Dashed yellow lines outline optic vesicle domains.

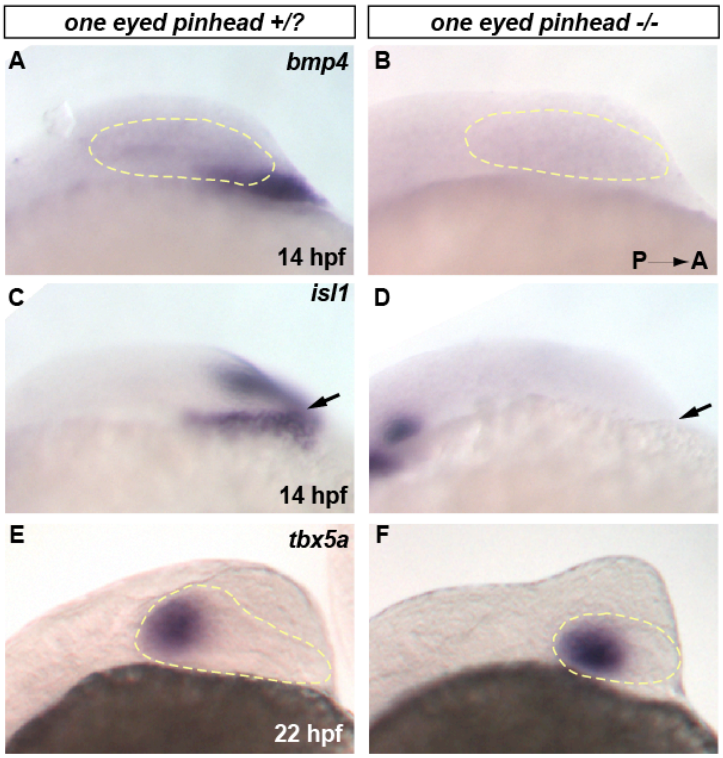

Figure 10. The prechordal plate, which expresses *bmp4*, is not necessary for dorsal retina initiation. (A-B) *One eyed pinhead* (*oep* ^{m134}) mutants do not initiate expression of *bmp4* in the prechordal plate region (C-D) *oep* mutants do not form prechordal plate tissues as assayed by expression of the prechordal plate marker *isl1* at 14 hpf (arrows in C and D). (E-F) However, dorsal retina develops normally in these mutants as assayed by expression of *tbx5a* at 22 hpf.

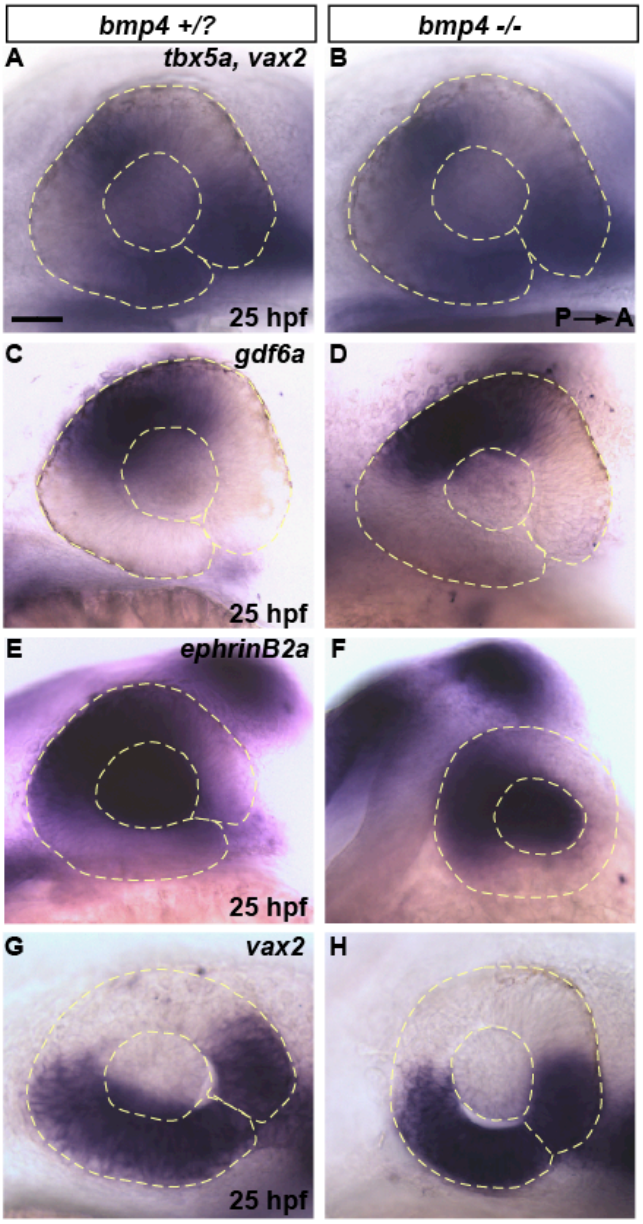

Figure 11. *bmp4* is not necessary for initiation or maintenance of dorsal retinal fate. (A-F) The dorsal-specifying genes *tbx5a*, *gdf6a*, and *ephrinB2a* are expressed normally in *bmp4*^{st72} mutants at 25 hpf, as is the ventral specifying gene *vax2*. (G-H) The ventral patterning gene *vax2* is also normally expressed in *bmp4* mutants at 25 hpf. mRNA expression of polarized dorsal and ventral genes (*tbx2a, tbx5a*, *gdf6a*, and *vax2)* is also initiated normally in *bmp4*^{st72} mutants at 12-15 hpf (data not shown). Scale Bar = 50 µm.

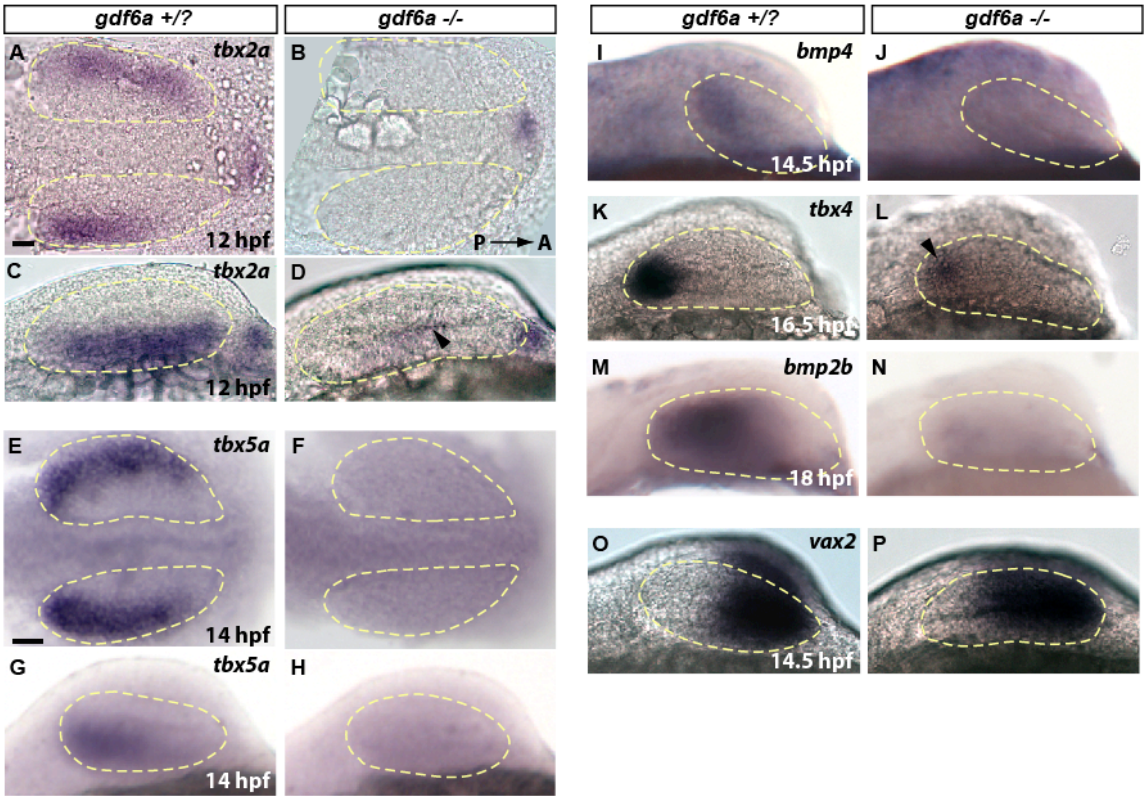

Figure 12. *gdf6a* is necessary for establishing dorsal retinal fate. In *gdf6a* mutants, dorsal retinal patterning genes either fail to express, or have greatly downregulated expression. Ventral genes are turned on normally, but expand shortly after to fill almost the entire retinal field. Expression of dorsally or ventrally expressed genes is shown at stages shortly after they are first detectable in wild type. (A-D) Robust 12 hpf *tbx2a* in *gdf6a* siblings is only faintly detectable in *gdf6a* mutants (arrowhead in D). (E-H) Expression of *tbx5a* begins at 12 hpf (14 hpf shown in figure) in sibling embryos, but is never seen in *gdf6a* mutants (F, H). (I-N) Similarly, *bmp4* and *bmp2b* are completely lost in *gdf6a* mutants, while *tbx4* expression is initiated at very low levels (arrowhead in L). (O-P) Expression of *vax2* is only slightly expanded at 14.5 hpf, and continues to expand to fill most of the retina by 24 hpf (See Figure 13 R) Anterior to the right; A, B, E, F are dorsal views; C, D, G-R are lateral views. Scale bars = 50 µm. Dashed yellow lines outline optic vesicles.

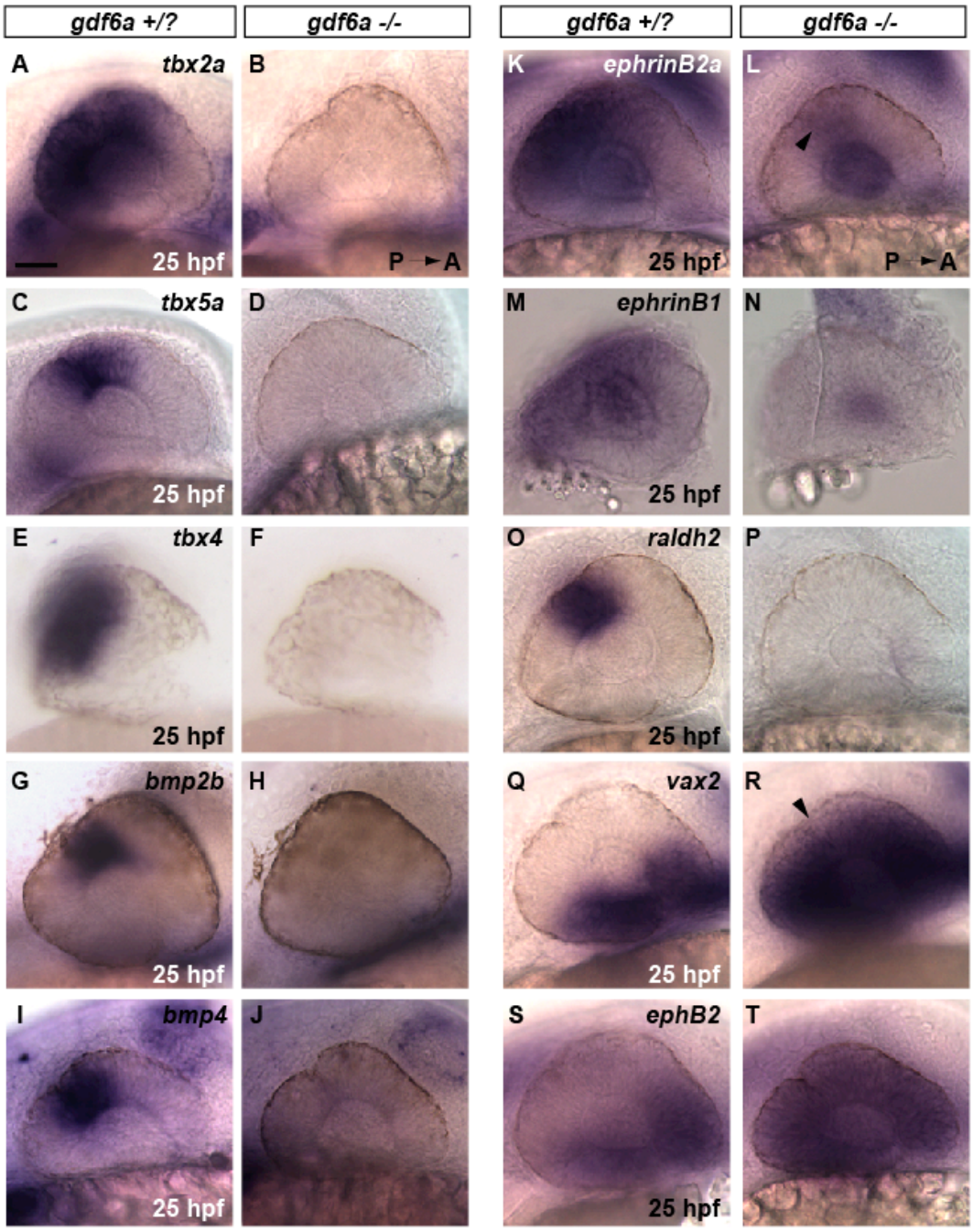

Figure 13. Gdf6a is necessary for maintenance of dorsal retinal fate. In *gdf6a* mutants, dorsal retinal patterning genes are absent or greatly downregulated at 25 hpf. Ventral genes are expanded to fill the majority of the retinal field. (A-P) Polarized dorsal 25 hpf expression of *tbx2a, tbx5a, tbx4, bmp2b, bmp4,ephrinB2a, ephrinB1 and raldh2* in *gdf6a* siblings is absent in *gdf6a* mutants or faintly detectable in the case of *ephrinB2a* (arrowhead in L). (Q-T) Expression of *vax2* and *ephB2* ventral markers is robustly expanded at 25 hpf in gdf6a mutants to fill most of the retinal field (arrowhead in R marks small portion without *vax2* expression). (A-T) Anterior to the right; lateral views. Scale Bar = 50 µm.

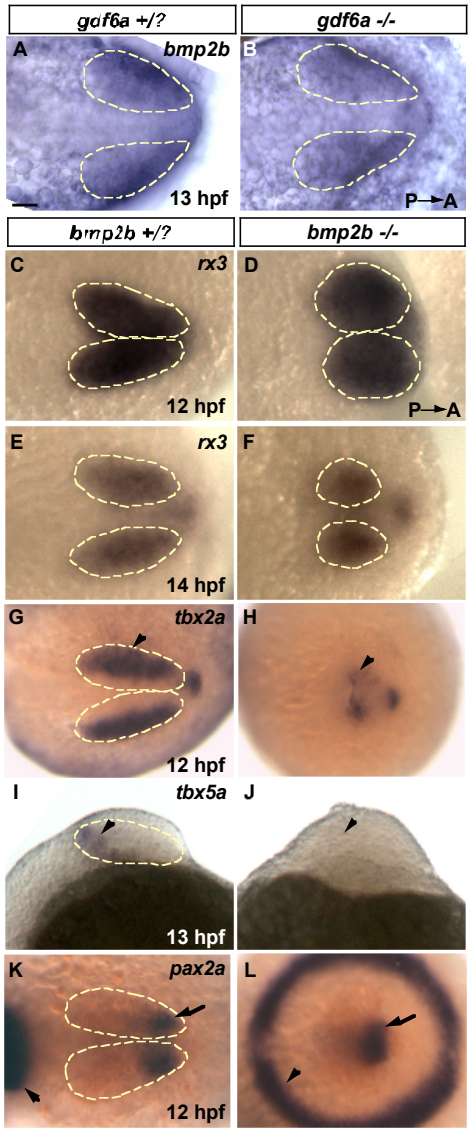

Figure 14. *bmp2b* is necessary for dorsal retina initiation. Despite severe morphological defects, *bmp2b* mutants develop *rx3* expressing eye fields. They fail, however to initiate expression of several dorsal markers. (A-B) *bmp2b* is expressed in the extraocular ectoderm in 13 hpf *gdf6a* siblings (A) and this expression domain is unchanged in *gdf6a* mutants (B). (C-F) *rx3* expression in siblings and *bmp2b* mutants at 12 and 14 hpf. (G, H) The earliest dorsal fate marker, *tbx2a*, is expressed in siblings beginning at 11 hpf (12 hpf shown, arrowhead). *tbx2a* is greatly downregulated in *bmp2b* mutants (H, arrowhead). (I, J) *tbx5a* expression is initiated at 12 hpf in siblings (13 hpf shown, arrowhead in I), but never turns on in *bmp2b* mutants (arrowhead in J where staining is absent). (K, L) In 12 hpf siblings, *pax2a* marks the prospective ventral retinal domain (*arrow*, K), which is also present in *bmp2b* mutants at this time point (*arrow*, L), despite severe dorsalization, which leads to a circular midbrain-hindbrain boundary (arrowheads; compare L with K). I-J are lateral views, anterior to the right; A-H, K-L are dorsal views, anterior to the right. Scale Bars = 50 µm. Dashed yellow lines outline optic vesicles where boundaries are visible.

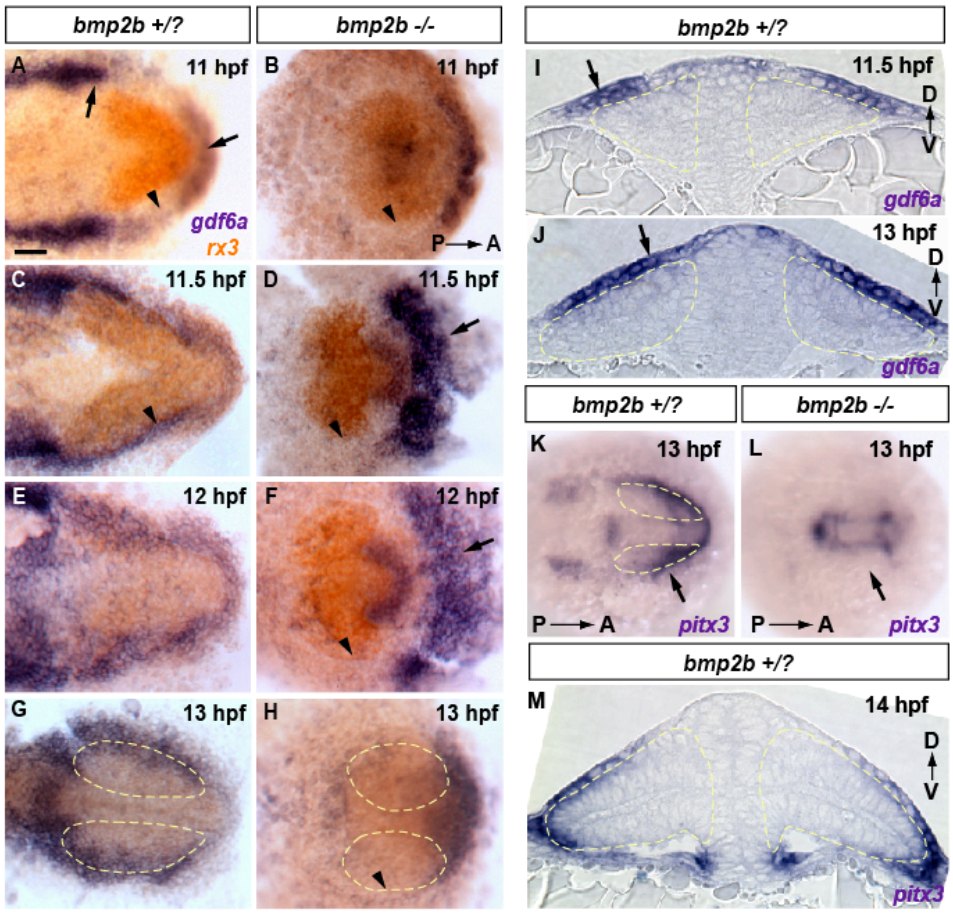

Figure 15. The dorsal initiation signal arises from non-neural ectoderm at 11-12 hpf. Extraocular *gdf6a* is expressed in the correct time and place to initiate dorsal fate within the retina, and expression is absent in *bmp2b* mutants, lacking dorsal fate. These results suggest that lateral head ectoderm is the tissue responsible for initiating dorsal fate within the retina. (A-H) *gdf6a* expression in blue, *rx3* expression in orange. (A, C, I) *gdf6a* in *+/?* siblings is expressed in the non-neural head ectoderm, and is initially strongest anterior and posterior to the optic vesicle (arrows in A, arrowhead in A marks weaker expression lateral to the optic vesicle). At 11.5 hpf *gdf6a* expression has intensified covering the lateral portion of the optic vesicle (C, arrowhead; I transverse section, arrow). (E, G, J) *gdf6a* later expands to cover the majority of the dorsal leaflet of the optic vesicle (G; J transverse section, arrow). (I, J) Extraocular *gdf6a* at 11.5-13 hpf is directly adjacent to optic vesicle *tbx5a*, initiated at 12 hpf (Compare I, J to Figure 7 A0-A3).

(B, D, F, H) In *bmp2b* mutants the lateral extraocular domain of *gdf6a* is completely absent (arrowheads in B, D, F, H). Expression of *gdf6a* anterior to the optic vesicle is upregulated in mutants (arrows in D and F). (K, L, M) The extraocular lateral domain of the non-neural ectoderm and lens placode marker *pitx3* is also absent in *bmp2b* mutants (arrows in K, L), indicating that *bmp2b* mutants do not form the non-neural ectodermal tissue necessary for *gdf6a* expression and subsequent initiation of dorsal fate. (A-H, K, L), whole mount dorsal view, anterior to the right. (I, J, M) transverse sections. Scale Bars = 50 µm. Dashed yellow lines outline optic vesicles.

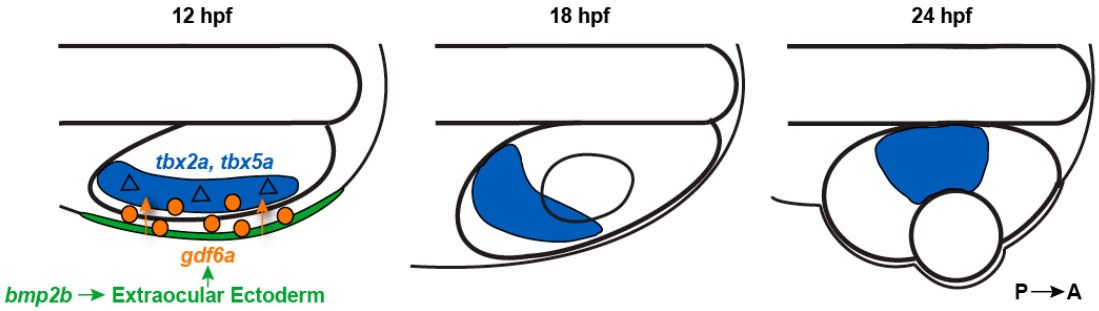

Figure 16. Lateral head ectoderm initiates dorsal retinal fate starting at 11 hpf. I propose that *bmp2b* acts upstream of *gdf6a* to pattern the non-neural ectoderm, the critical tissue for initiating dorsal retinal fate. *gdf6a* expressed in the non-neural ectoderm (prospective lens placode) during optic vesicle evagination, acts to initiate the expression of prospective dorsal markers within the lateral presumptive retinal field. The first polarized prospective dorsal markers, *tbx2a* and *tbx5a*, turn on at 11 and 12 hpf respectively (12 hpf shown in model) in the dorsolateral leaflet of the optic vesicle. Many cells expressing these dorsal markers undergo morphogenesis to populate the dorsal and central retina at 24 hpf.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL ZEBRAFISH MUTANT, *BIG TOP* (*BTP^{ZC52}*), INVOLVED IN DORSAL-VENTRAL RETINAL PATTERNING

Abstract

The patterning of retinal cells in both anterior-posterior and dorsal-ventral axes is necessary for formation of axon guidance molecule gradients within retinal tissue. These guidance molecule gradients then orchestrate retinotopic projections of retinal ganglion cell axons to their brain targets, and are thus necessary for the formation of the topographic neural map required for visual processing. I performed a forward genetic screen in zebrafish to identify novel mutations in genes with required roles in specifying the retinal dorsal-ventral axis. I used a cocktail of *in situ* hybridization probes directed against the transcription factors *tbx5a* (a marker of dorsal retina) and *vax2* (a marker of ventral retina), and screened for altered expression of one or both of these genes in potential F3 mutants. I identified, mapped, and characterized a novel mutant named *bigtop* (*btp^{zc52}*), which shows expanded dorsal *tbx5a* expression and reduced ventral *vax2* expression. *btp* mutants also show expanded expression of three dorsal *bmp* family genes, delayed retinal development, and abnormal retinotectal projections. The mutant gene was mapped to a four-megabase region on chromosome two. Experiments to clone the gene are ongoing.

Introduction

Projections of retinal ganglion cell (RGC) axons to the optic tectum (or superior colliculus in mammals) form a retinotopic map, where neighboring RGCs project their axons to neighboring target zones in the brain (Luo and Flanagan, 2007). Development of these accurate axon projections requires patterning of source (retina) and target (tectum) tissues along two perpendicular axes (McLaughlin et al., 2003). In zebrafish, the retina is patterned across both dorsal-ventral (D-V) and anterior-posterior (A-P) axes, as evidenced by the numerous genes expressed in polarized D-V and A-P domains. Polarized gradients of gene expression along two axes within a planar tissue can establish a unique positional identity, and allow for accurate axon targeting. Axial patterning within a tissue, such as the retina, can be initiated by morphogen expression, which regulates expression of transcription factor genes in one half of the axis (e.g. dorsal). These transcription factors can then regulate polarized expression gradients of axon guidance molecules, leading to precise targeting of neurites (McLaughlin and O'Leary, 2005; Harada et al., 2007).

I envision D-V patterning occurring in a genetic hierarchy, with different morphogens, transcription factors, and axon guidance genes acting to initiate, maintain, and refine the graded fates of individual RGCs along the D-V axis. An ultimate goal is to identify and characterize all of the molecules responsible for patterning the dorsal-ventral axis of the vertebrate retina. There are many genes with clearly described polarized D-V expression patterns in the vertebrate retina. Gene families with expression concentrated at the dorsal pole include *bmps*, *wnts*, *tbxs*, and *ephrins*, and ventrally expressed genes include *shh*, *bmp*-inhibitors, *vax2* and *ephs* (Thisse, 2001; Thisse, 2005). However, the

role that many of these genes play in the retinal-patterning cascade is unclear because a significant portion have not been studied in true loss-of-function experiments. Complicating the analysis, in the mouse, an organism in which true loss of function experiments can be performed, D-V patterning candidate-gene mutants, including *Bmp4*, *Tbx5*, and *EphrinB2* knockouts, die before retinal-patterning phenotypes can be analyzed (Wang et al., 1998; Adams et al., 1999; Bruneau et al., 2001; Murali et al., 2005).

Therefore, many of the proposed D-V patterning genes have only been tested using gain-of-function experiments (McLaughlin et al., 2003). These misexpression experiments can be difficult to interpret because abnormally high levels of protein can lead to non-specific effects, especially between members of a gene family. As one example, five *tbx* family transcription factor genes are expressed in overlapping patterns dorsally, therefore phenotypes observed following overexpression of one of these genes might actually be due to the function of another (Koshiba-Takeuchi et al., 2000). Hence, while many genes are hypothesized to be important in D-V patterning, the functions of these genes and their placement in the genetic tissue patterning pathways leading to D-V identity are unknown.

Zebrafish are a powerful model organism for studying retinal development. Zebrafish eyes develop quickly, eyes are large, and retinotectal mapping is precise from an early stage. However the library of retinal patterning null mutants is far from complete. Morpholinos are commonly used to knock down expression of genes in zebrafish, but this approach can be problematic due to incomplete knock down as well as non-specific effects on gene expression. Forward genetic screens are an unbiased approach for identifying genes required for a process of interest. Because phenotypes are

identified first, problems with redundancy are avoided, and the resulting mutant alleles recovered allow for superior loss of function experiments to morpholinos. In zebrafish there have been no previous screens looking specifically for D-V patterning genes and only three mutants afftcting D-V patterning have been found previously in unrelated screens (Trowe et al., 1996; Muto et al., 2005), suggesting that screens have not identified all genes in the D-V patterning pathway.

I performed an F3 forward genetic screen in zebrafish to generate new mutations in genes required for D-V retinal patterning. My screen was designed to identify genes important during all stages of D-V axis specification, including those involved in initiation, maintenance, and refinement of D-V fate. Most importantly, I expected that not all genes important for specifying the D-V axis are known, and therefore my screen would identify mutations in previously uncharacterized genes. Additionally, since no zebrafish mutants currently exist for genes, including several *tbxs*, *wnts*, and *ephs* with known or expected roles in D-V patterning, I also hoped to identify mutations in some of these genes.

My F3 *in situ* hybridization screen assay specifically addressed patterning in the D-V axis. I used an *in situ* probe cocktail directed against two transcription factors, *tbx5a* (dorsal retina) and *vax2* (ventral retina), and screened for altered expression of one or both of these genes. I expected that mutations altering the retinal expression pattern of the dorsal transcription factor *tbx5a* and/or the ventral transcription factor *vax2* would affect genes required for establishing dorsal-ventral retinal polarity. I hypothesized that transcription factors act between morphogens and axon guidance molecules in D-V axis specification, and therefore had the potential to identify mutations in upstream genes that

positively regulate their expression, as well as downstream genes whose loss of feedback regulation would alter their expression. Furthermore, transcription factors are expressed in relatively precise domains, rather than in gradients, as with morphogens and guidance molecules, therefore changes in their expression are easier to detect. Genetic screens in zebrafish are not a trivial undertaking; therefore we designed a model in which four labs shared the burden of work.

I identified three novel mutants in my screen with altered expression of D-V retinal patterning markers: *bigtop*^{zc52}, *froggy*^{zc54}, and (un-named) *3*^{zc53}. After an initial characterization of all three mutants, I decided to continue experiments only with the *bigtop* mutant. I analyzed the retinal patterning phenotype of *bigtop* mutants, and found it results in expansion of dorsal markers and reduction of ventral markers. *bigtop* does not have a role in initiation of dorsal fate, but rather functions in regulating the maintenance of D-V patterning. I also show that the *bigtop* mutants have delayed retinal development and abnormal retinotectal projections. I contracted the company Floragenex to map the *bigtop* mutation to a four-megabase region on chromosome two. Experiments to clone the *bigtop* gene are ongoing.

Methods

Animals

I maintained adult zebrafish (*Danio rerio*) on a 14-hour light, 10-hour dark cycle. I raised embryos in E3 with methylene blue at 28.5 °C, anesthetized with 0.2mg/ml tricaine and fixed in 4 % paraformaldehyde, unless otherwise noted. I staged embryos

according to (Kimmel et al., 1995), by counting somites (morphologically) and/or by *in situ* using the somite marker *myoD*.

Mutagenesis and creation of F2 families

Tübingen strain wild-type lethal-free adult males were mutagenized with N-ethyl-N-nitrosourea (ENU) using standard techniques (Westerfield, 2000). They were outcrossed to non-mutagenized females to create an F1 founder generation, heterozygous for randomly distributed genetic mutations, which are distinct in each individual. Individual F1 females were then crossed to individual F1 males to double the mutational load, creating F2 families, heterozygous for new mutations throughout the genome. Pairs of F2 sibs were then incrossed to homozygose the new mutations in ¼ of the progeny expected in ¼ of the crosses. F3 clutches were collected, and living, fertilized embryos are sorted at 3-5 hours postfertilization (hpf). At completion, I had screened four or more F3 clutches from 64 F2 families. Spawning rates were not always high within F2 families; therefore I also screened 1-3 clutches from families with low spawning rates, in addition to the 64 described above.

Screening assay

F3 clutches were raised to 24 hpf, dechorionated with pronase, and fixed. We optimized a *tbx5a/vax2 in situ* hybridization probe cocktail for screening. I developed a protocol for performing 30 *in situ* hybridizations in parallel, and were able to screen ~30 F3 clutches per week.

Mutant Recovery, Mapping, and Cloning

Mutant Recovery: I outcrossed putative mutant *bigtop* F2 fish with wild type Tübingen fish, and verified the mutant phenotypes, observed in 25% of embryos from 25% of next generation F3 incrosses. I cryopreserved sperm and saved carriers to establish mutant lines. Map cross details: A map cross was made between a *btp/Tü* heterozygous Tübingen male and a *WIK/WIK* female to obtain *btp/WIK*, and *Tü/WIK* offspring. These were grown to adulthood and next generation heterozygotes were identified by random incrosses and observation of the four day postfertilization (dpf) mutant phenotype in one quarter of the offspring from a cross of two heterozygous parents. *btp/WIK* adults were incrossed to obtain *WIK/WIK*, *btp/WIK*, and *btp/btp* embryos, which were separated at 4 dpf by morphological phenotypes into sibling and mutant pools. Preparation of DNA for Floragenex: I prepared pooled DNA form 40 sibling (*WIK/WIK*, *btp*/WIK) and 40 mutant (*btp/btp*) four-dpf map cross embryos. DNA was prepared using the Qiagen DNeasy Blood and Tissue Kit and analyzed for quantity and purity on the Qubit Fluorometer. This genomic DNA was then sent to Floragenex for mapping using the RAD tag technique (Figure 17 A). Floragenex Mapping: Floragenex analyzes all chromosomes for a region with a higher incidence of *WIK* DNA in the sibling pool compared to the mutant pool. Briefly, DNA is digested with SbfI, an 8 base pair cutter restriction enzyme, ligated with an adaptor, and sequenced. Restriction site associated DNA is hybridized to microarrays, and mutations are mapped to a chromosomal region (1-10 cM), using a bulk segregant mapping approach. Preparation of RNA for RNA sequencing: Total RNA for RNA sequencing was prepared from 20 pooled mutant or sib 2.5 day postfertilization (dpf) embryos. These embryos were obtained from F5 *bigtop/+* adult incrosses (F5 -

indicating five generations of Tübingen wild-type strain outcrosses after ENU mutagenesis, and no contamination of DNA polymorphisms from other strains). Fresh tissue was shredded using 21.5 gauge needles in Qiagen RLT buffer from the RNeasy kit. These shredded tissue samples were run through Qiashredder columns before prepping total RNA following the Qiagen RNeasy kit protocol. Total RNA was analyzed for quantity and purity by the microarray core before creation of cDNA libraries and Illumina RNA sequencing on mutant vs. sibling pools. Bioinformatic analysis of RNA seq data: Bioinformatic analysis on mRNA sequencing data was carried out by Brett Milash in the University of Utah Huntsman Cancer Institute bioinformatics core, to identify SNPs that are predicted to alter amino acid sequence in the mutant pool, as compared to the sibling pool and the zebrafish reference genome.

btp/+; isl2b:GFP transgenics

I outcrossed heterozygous *bigtop* and *froggy* adults with *isl2b:GFP* transgenics (Pittman et al., 2008) to analyze retinotectal axon projections of mutants. The *isl2b:GFP* transgene labels retinal ganglion cells and their axon projections to the tectum. This transgene also labels trigeminal neurons, Rohon Beard cells, as well as several other cell populations.

In situ hybridization

I performed whole mount *in situ* hybridization to analyze mRNA expression as described by (Thisse and Thisse, 2008) with the following modifications: I incubated and washed some of the samples using a Biolane HTI *in situ* hybridization machine (Huller and Huttner AG, Tübingen, Germany), and followed the technical modifications

described in the results. I synthesized Digoxigenin-UTP labeled riboprobes using in vitro transcription RNA labeling kits from Roche. Probes were as follows: *tbx2a* (Dheen et al., 1999), *tbx5a* (Ruvinsky et al., 2000), *bmp4* (gift, M. Mullins, University of Pennsylvania), *bmp2b* (Nikaido et al., 1997), *vax2* (Take-uchi et al., 2003), *gdf6a* (Veien et al., 2008), *ephrinB2a* (Durbin et al., 1998), *myoD* (Weinberg et al., 1996). Probes were developed with BM-Purple. I cleared whole mount embryos in 50-80% glycerol and imaged them using an Olympus SZX 12 stereomicroscope, an Olympus SN1H045411-H camera, and Picture Frame™ imaging software version 2.3. Sectioned embryos were embedded in plastic according to (Sullivan-Brown et al., 2011), and sectioned transversely at 12 μm thickness using a Reichert-Jung 2050 microtome. All sections were imaged on an Olympus BX51WI inverted microscope using the same camera and imaging software as for whole mount images. Toluidine Blue dye was used for visualizing tissues in plastic sections.

Results

Isolation of three genes required for D-V patterning

I carried out an F3 mutagenesis *in situ* hybridization screen in zebrafish to identify genes required for dorsal-ventral retinal patterning. *tbx5a* and *vax2* transcription factor probes were chosen for the *in situ* assay because of their clearly polarized and non-overlapping expression domains along the dorsal-ventral (D-V) axis of the retina and their known importance for D-V retinal patterning (Koshiba-Takeuchi et al., 2000; Barbieri et al., 2002; Mui et al., 2005). I recovered three novel D-V patterning mutants from this screen: *bigtop*^{zc52}, *froggy*^{zc54}, and *3*^{zc53}, and performed an initial characterization

on each mutant. Specifically, I analyzed the morphological development of mutants through five days postfertilization (dpf), determined the expression of D-V patterning genes *tbx5a*, *bmp4*, *vax2*, and *pax2a*, during early (14 hpf) and late (24 hpf) phases of D-V axis specification; and characterized retinotectal architecture by crossing the mutations into fish with the *isl2b:GFP* transgene.

zc54 was isolated due to a loss of *vax2* and expansion of *tbx5a*. However, this 24 hpf retinal patterning phenotype is not fully penetrant (Figure 18 A-B). I named this mutant *froggy*, because embryos bear a resemblance to *Xenopus* embryos (Figure 18 C-G), having a small eye and protruding lens (Figure 18 E, G). *froggy* mutant retinas show rapidly progressing tissue disorganization and cell death (Figure 19). Mutant lenses appear normal (Figure 19 D, F), however, the neural retina does not maintain its cup shape around the lens at later time points (Figure 19 F). Initiation of D-V retinal patterning markers at 12-15 hpf is normal in *froggy* mutants (data not shown). Furthermore, despite severely disorganized retinas and small eyes (Figure 19), these mutants form a surprisingly normal retinotectal projection (Figure 20). *zc53* has widespread necrosis at 24 hpf, and dies by 2.5 dpf. Therefore, retinotectal projections cannot be analyzed in these mutants. Expression of D-V patterning markers is normal at initiation time points in *zc53* (data not shown). After cryopreserving sperm from *froggy*^{zc54} and *mutant 3*^{zc53} I did not continue to characterize these mutants, and I turned my focus to the *bigtop* mutant.

<u>*bigtop* is necessary for D-V retinal patterning</u>

bigtop^{zc52} mutants have a fully penetrant retinal patterning phenotype, with expanded *tbx5a* and diminished *vax2* compared to siblings (Figure 17 A-B). While fully

penetrant, this phenotype has variable severity between mutant individuals. Some individuals have a domain of *vax2* that is almost the same size as the controls, but with lower expression, whereas others almost completely lack *vax2* expression. Similarly, both domain expansion and increased intensity of *tbx5a* expression are seen in mutants, that also varies between individuals (variability data not shown). The expression domains of the *bmp* family morphogens *gdf6a*, *bmp4* and *bmp2b* are also significantly expanded in *bigtop* mutants at 24 hpf (Figure 17 C-H). These defects are fully penetrant, and less variable than the expression of *tbx5a* and *vax2* transcription factors. All genes I analyzed, including *tbx5a*, *tbx2a*, *tbx4 gdf6a*, *bmp4*, and *bmp2b* dorsally, and *vax2* and *ephB2* ventrally show normal expression at their time of initiation, suggesting that *bigtop* affects the maintenance of D-V patterning, but not its initiation. Additionally, I performed a detailed time course analysis to determine what time dorsal markers first showed expansion compared to control sibling embryos. I analyzed the expression of dorsal genes *tbx5a*, *tbx4 bmp4*, and *bmp2b* at 14, 16, 18, 21, 28, and 44 hpf (data not shown). I found that expression of dorsal genes begins to expand between 16-18 hpf, and expression of all genes tested remains robustly expanded after 18 hpf, through 44 hpf, my last time point assayed.

<u>*bigtop* mutants have altered morphological development</u>

bigtop mutants have altered morphological development (Figure 21 A). Small eyes and less pigmentation are observed in mutants beginning at two dpf. Mutants develop a heart edema, and never develop an obvious swimbladder. Mutants display a twitching behavior at three dpf, appearing to have repeated spontaneous seizures (data not shown). At four dpf mutants have an abnormal visual background adaptation (VBA)

phenotype. VBA is a stereotyped cellular response in which melanophores restrict upon exposure to bright light. This camouflaging adaptation is thought to be due to projections of dorsal RGCs to the hypothalamus (Roeser and Baier, 2003). In *btp* mutant animals melanophores do not constrict in response to bright light at four dpf (Figure 21 A; see yolk and ventral tail compared to sibling).

Retinal development is abnormal in *bigtop* mutants

Histological sections stained with toluidine blue reveal that *btp* mutant retinas are slightly smaller at 24 hpf, but optic cup formation and cellular morphology appear normal (Figure 21 B-C). At two dpf, however, retinal development appears strongly delayed compared to siblings (Figure 21 D-E). In two dpf wild-type siblings, retinal layers are visible, though not fully developed, and the optic nerve of retinal ganglion cell axons exiting the eye is apparent (Figure 21 D, arrow marks optic nerve). However, in mutants, the cells of the retina remain undifferentiated and no layers are visible, indicating a clear delay in development (Figure 21 E). By three dpf, all retinal layers are present in mutants (Figure 21 G), and a clear optic nerve is present (arrow in Figure 21 G), however the eye is significantly smaller than controls, and morphologically, eyes appear at least one day delayed in development (compare Figure 21 G with Figure 21 D). A significant undifferentiated ciliary marginal zone (CMZ) remains present dorsally in *btp* mutants (Figure 21, arrowhead and dashed line in G), yet this zone appears absent ventrally in mutants (Figure 21 G, open arrowhead. Again compare arrowheads and yellow outline of CMZ in siblings, Figure 21 D). The retinal layers also appear slightly more disorganized than in siblings but this is closer in appearance to the two dpf sibling retina (Figure 21 F-G, again compare G with D versus F).

bigtop is necessary for retinotectal axon targeting

I analyzed retinal ganglion cells development and their axon projections in *bigtop* mutants compared to siblings using the *isl2b:GFP* transgene (Figure 22). In sibling five dpf larvae, retinotectal axon projections display clear dorsal and ventral optic tracts. Tectal lobes are robust, and axon arborizations evenly fill the tectal neuropil (Figure 22 A). In mutants, I observed significantly less axon innervation in the tectum, and in particular less axon branching in the anterior tectum (Figure 22 C, E). After reaching the tectum, many axons in mutants project toward the midline and posterior body. The neurites leave the typical tectal neuropil region and extend to the borders of the tectum (open arrowheads in Figure 22 C, E). Additionally, in a small percentage of mutants, some axons misproject ipsilaterally (arrow in Figure 22 E), leaving the contralateral tectum with very few axons. The tectal neuropil and full tectal area appear smaller in mutants (compare neuropil area in C and E with A). Axon projection phenotypes are variable between individuals (compare Figure 22 C with E). Numbers of axons reaching the tectum, and number of axons overprojecting varies between individuals.

bigtop mutant mapping and RNA sequencing

Contracting with Floragenex, a company that provides zebrafish DNA mapping services, located in Eugene, Oregon, I mapped the *bigtop* mutation to chromosome two (Figure 23). *btp/WIK* map cross adults were incrossed to obtain *WIK/WIK*, *btp/WIK*, and *btp/btp* embryos, which were separated at four dpf by morphological phenotypes into sibling and mutant pools. DNA was prepared from each of these pools of embryos, and sent to Floragenex for mapping (Figure 23 A). Briefly, Floragenex analyzed pooled DNA, screening DNA for a chromosomal area with a higher incidence of *WIK* DNA

(compared to Tübingen DNA) in the sibling pool. Because my F3 screen was performed in the Tübingen background, I expect that markers closely linked to the *bigtop* mutation would be from the Tübingen strain. Floragenex identified that chromosome two contained an average number of linked markers significantly above background rate (doted red line), indicating a higher incidence of *WIK* strain DNA in the sibling pool than expected by chance alone (Figure 23 B). Therefore, chromosome two is enriched for Tübingen DNA in mutant pool, showing that chromosome two is linked to the *bigtop* mutation. The segregation of individual Floragenex RAD tags from chromosome two identified a four megabase region completely linked to the *btp* mutation. This region is located between 42-46 million base pairs on chromosome two (in the Sanger zebrafish zv8 reference genome) (Figure 23 C), and between 43-47 million base pairs on chromosome two (in the zv9 reference genome) (Table 1).

This region of chromosome two contains 92 annotated genes, and while many have known retinal expression, many others also have no known function or expression data (Table 1). The best candidate gene for the mutation located in this region, is the receptor typrosine kinase family *ephB1* axon guidance receptor. *ephB1* was not annotated in the zebrafish zv8 genome version, therefore it was not originally identified as a candidate gene for *bigtop* following Floragenex mapping. I have not yet shown that *btp* is a mutation in *ephB1*, and experiments to rule out this candidate are ongoing.

High throughput Illumina mRNA sequencing was carried out by the University of Utah Microarray Core on cDNA libraries prepared from total RNA from 2.5 dpf pools of 20 *btp* sibling and 20 *btp* mutant individuals per pool. Bioinformatic alignment and analysis of mutant mRNA sequences compared to sibs and the zebrafish genome

reference sequence was done by the University of Utah Bioinformatics Core. I hoped to find nonsense or missense mutations within mRNAs from the region of chromosome two identified by Floragenex as carrying the *btp* mutation. While I focused my variant detection on this region, I have not yet found any SNP variants in the mutant pool that create stop codons or nonsynonymous base pair changes leading to critical amino acid substitutions in candidate genes.

Discussion

I performed an F3 forward genetic screen to identify mutations in novel and candidate dorsal-ventral (D-V) patterning genes. I hypothesized that mutations affecting the expression patterns of the D-V transcription factor genes *tbx5a* and/or *vax2* would likely be in genes necessary for establishing retinal D-V polarity and topographic retinotectal projections. I hoped to identify mutants with dorsalized or ventralized retinas, and I expected that these mutations would affect D-V gene expression at one of three stages: initiation, maintenance, or refinement. In my small-scale screen, I recovered three novel mutations affecting D-V patterning. Two of these, *froggy*^{zc54} and *zc53*, likely affect retinal patterning non-specifically, through other effects on retinal development. The most interesting mutation, *bigtop* (*btp*^{zc52}), is characterized by a dorsalized retinal patterning phenotype. Dorsal markers are expanded in *btp* mutants and ventral markers are restricted. I found this patterning phenotype affects the maintenance of expression of D-V patterning genes, but not their initiation. These results suggest that *btp* encodes a protein important for maintaining ventral retinal fate. *bigtop* was mapped to a four megabase region on chromosome two that contains the *ephB1* gene. This gene is an excellent candidate gene for the *btp* mutation because it has known expression in the

ventral retina in other species including mouse (Williams et al., 2003; McLaughlin and O'Leary, 2005) and *Xenopus* (Smith et al., 1997), and its ventrotemporal expression in the mouse retina is known to regulate guidance of ventrotemporal RGC axons to project ipsilaterally instead of contralaterally (Williams et al., 2003; Chenaux and Henkemeyer, 2011).

EphB's are members of the receptor tyrosine kinase family of membrane bound axon guidance receptors, and respond primarily to the EphrinB ligands. In zebrafish, *ephB1* was only recently annotated in the current genome assemblies, and to my knowledge neither function nor expression of this gene has been previously analyzed. However, other members of the EphB receptor family have known expression in high ventral to low dorsal gradients in the zebrafish retina, and are hypothesized to regulate D-V topographic mapping (McLaughlin et al., 2003; McLaughlin and O'Leary, 2005; French et al., 2009). If *btp* is indeed a mutation in the *ephB1* gene, this will be the first example, to my knowledge, of an Eph regulating tissue patterning.

In addition to the retinal patterning phenotype, the *btp* mutation also causes a delay in development of the neural retina and lens, and altered retinotectal axon projections. Axons in the tectum of *btp* mutants both overshoot the normal area of tectal neuropil, as well as misproject ipsilaterally. Additionally, axons appear to arborize preferentially in the posterior tectum. My attempts to assay D-V specific retinotopic axon targeting have been unsuccessful thus far. Dye injections have been difficult to perform and interpret, likely due to the very small size of *btp* mutant eyes at five dpf. However, I have also attempted to analyze dorsal axon targeting in mutants using the dorsal-specific *bambib:gal4* transgenic identified in a *gal4* gene trap screen by Hideo Otsuna in our lab

(unpublished results). *bambib* encodes the bmp and activin membrane bound inhibitor, a gene with known dorsal retinal expression When crossed with *UAS:tagRFP-CAAX* transgene, *bambib:gal4* drives expression of RFP in a small subset of dorsal retinal ganglion cells, allowing their axon targeting to the ventral tectum to be imaged on a confocal microscope. I crossed the *bigtop* mutation into the *bambib:gal4* line, and can clearly visualize dorsal retinal ganglion cells labeled with *tagRFP-CAAX*; however, I have never observed axons in the tectum using this line. Either dorsal axons never reach the tectum in *btp* mutants, or the subset of labeled axons in *bambib:gal4*; *btp* mutants is too sparse to be visualized with the current techniques and fluorescent dyes.

Unfortunately, my attempts to identify the *bigtop* mutant gene via RNA sequencing have been unsuccessful thus far. Bioinformatic analysis of the sequence data has not identified any nonsense changes in the mapped region on chromosome two, and no missense changes in candidate genes. Potential problems with this approach include the age of embryos used for sequencing, because mRNA of the candidate gene must be present for it to be analyzed. Secondly, the gene may have highly restricted expression, and therefore low levels of mRNA within the embryo, leading to a absence in sequence data. Lastly, mapping to a small interval is critical if the mutation is not a nonsense mutation. Many missense mutations are expected, and it can be hard to tell if a given amino acid is critical for function of a gene without extensive testing. Despite my lack of success thus far, I will continue with my attempts to clone the *btp* mutation, including RT-PCR and *in situ* expression analyses of interesting candidate genes such as *ephB1*, and further analysis of the RNA sequencing data.

Some caveats exist to my forward genetic screen approach for identifying novel genes required for D-V patterning. For example, it is possible that some genes important for establishing dorsal-ventral retinal polarity are redundant, thus making identification in a recessive screen difficult. In support of this possibility is the known expression of multiple genes within a gene family in dorsal retina. Gene redundancy may be a fundamental reason why D-V patterning has been difficult to study. At least five T-box transcription factor genes, and five *bmp* family genes all have known overlapping expression in the dorsal retina in zebrafish. It is possible that the functions of genes within each of these families are at least partially redundant with one another, and this could lead to mutations in these genes having no or very mild phenotypes. If this is the case, double or triple mutants will be needed for uncovering the required functions of these genes in D-V patterning. Additionally, if full or partial redundancy of gene function is prevalent in D-V retinal patterning, reverse genetics approaches may be a more appropriate approach for studying this process. An alternative approach for identifying novel candidate genes expressed in polarized patterns across the D-V axis would be to perform a microarray screen comparing gene expression in dissected dorsal vs. ventral retinal tissue.

In summary, my forward genetic screen in zebrafish identified a novel genetic mutation, *bigtop,* which regulates dorsal-ventral patterning. No previous zebrafish mutation has this phenotype to my knowledge. Characterization and cloning of this mutation are ongoing, and knowing the identity of the *bigtop* mutation may give me new insights into the function of this gene and what role it plays D-V patterning.

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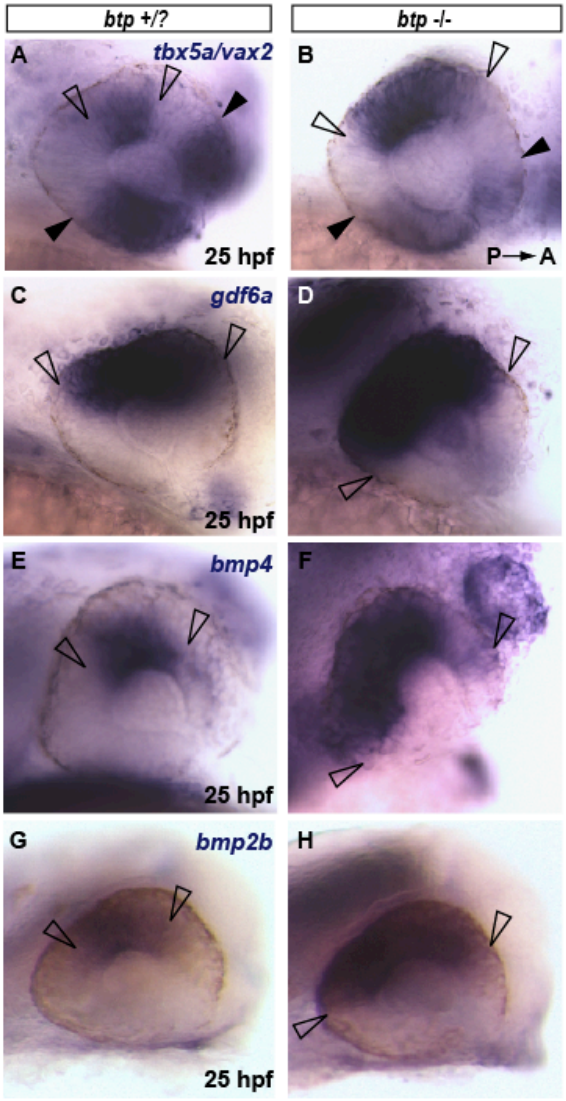


Figure 17. *btp* is required for dorsal-ventral retinal patterning. Expression domains of dorsal retinal patterning genes as visualized by *in situ* hybridization (blue) are expanded in *bigtop* mutants at 25 hpf. Arrowheads mark the outer limits of *in situ* staining in each retina. Open arrowheads for dorsal genes, closed arrowheads, ventral *vax2*. All images are whole mount, lateral views, anterior to the right. (A-B) mRNA expression of the T-box transcription factor *tbx5a* is restricted to a small domain at the dorsal pole (open arrowheads), directly opposite the choroid fissure in *btp* siblings and WT embryos (A). This domain is expanded to almost three times the WT size in *btp* mutants (B). The ventral marker *vax2* has a smaller expression domain (arrowheads) and decreased staining intensity in *btp* mutants compared to siblings. (C-H) *bmp* family genes *gdf6a*, *bmp4*, and *bmp2b* all show polarized dorsal mRNA expression in WT, and expression domains are significantly expanded in *btp* mutants. In the case of the three *bmp* genes tested, this domain expansion causes these dorsal markers to cover the whole dorsal half of the retina.

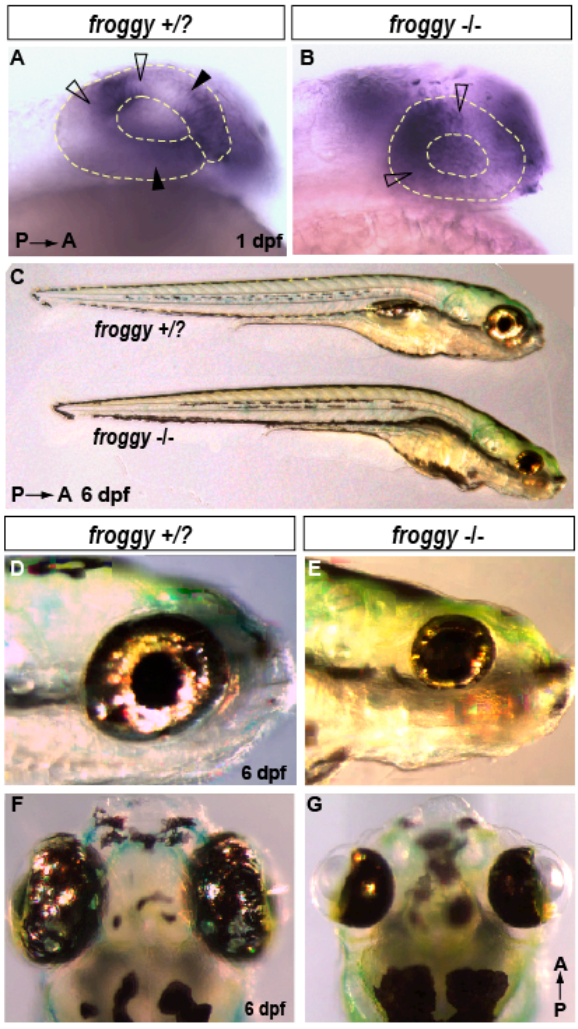


Figure 18. *froggy* mutant retinal patterning and morpholgy. (A-B) *froggy* mutants show complete loss of *vax2* and expansion of *tbx5a* dorsally compared to siblings, but this phenotype is not fully penetrant. Open arrowheads mark outer boundaries of *tbx5a*, closed arrowheads mark outer boundaries of *vax2* in A, *vax2* absent in B. (C-G) Morphologically, froggy mutants have a small eye and protruding lens (G) as well as a smaller head than siblings.

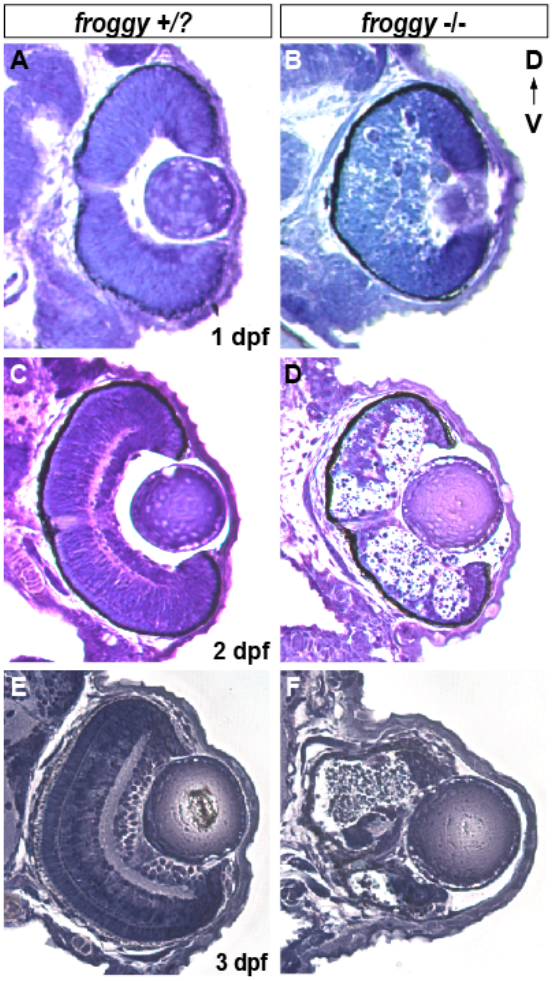


Figure 19. *froggy* mutants have abnormal retinal development. (A) At one dpf, the optic cup has formed around the lens in WT siblings, and undifferentiated retinal cells are evenly distributed throughout the optic cup. (B) In *froggy* mutants, by contrast, retinal cells are beginning to degenerate in some areas of the optic cup (representative section shown). Additionally, mutant eyes appear smaller in cross section, and the retinal-pigmented epithelium (RPE) appears thicker than in siblings. (C) At two dpf, sibling retinas contain multiple differentiated cell types, and an initial laminar structure is observed. Additionally, a retinal nerve of retinal ganglion cells exiting the eye is visible. (D) Two dpf mutant siblings show dramatic retinal decay. No differentiated cell types are clearly visible in these retinas, and no laminar structure is observed. However, an optic nerve is visible, indicating the presence of retinal ganglion cells and axons exiting the eye. Additionally, the RPE appears normal, the optic cup has relatively normal morphology, and the lens appears normal. (E) Three dpf sibling retinas have many differentiated cell types, and well developed layers. Lens cells are differentiated at three dpf. (F) *froggy* mutant retinas show dramatic degeneration at this time point. Again, individual differentiated cell types are not visible in mutants, and the optic cup morphology is dramatically different from siblings. Still, an optic nerve is visible in mutant retinas. Furthermore, the lens tissue is normal size and contains differentiated cell types, as in siblings.

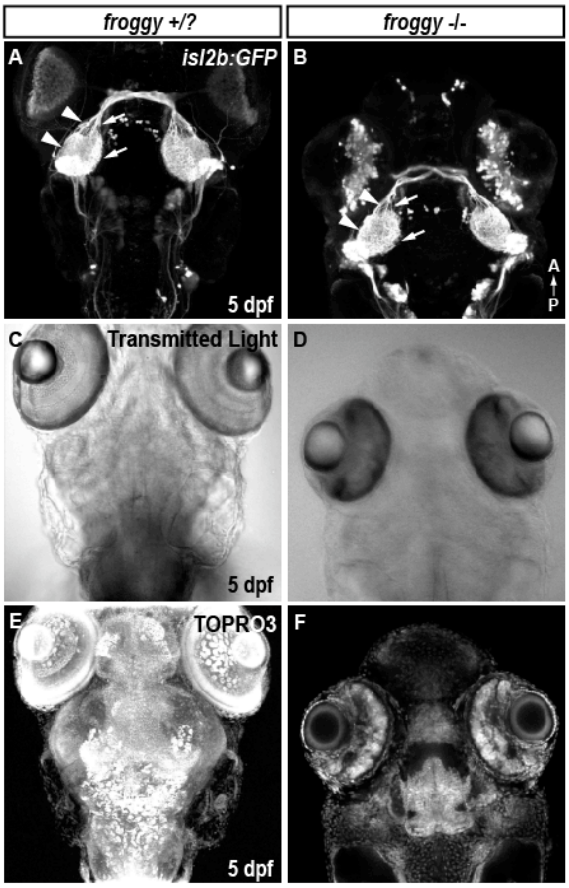


Figure 20. Retinotectal projections appear normal in *froggy*. (A) Retinotectal axon projections in sibling embryos visualized using the *isl2b:GFP* transgene display clear dorsal (medial) and ventral (lateral) optic tracts and tectal lobes (arrows mark dorsal tract and tectal lobe, arrowheads mark ventral tract and tectum). Axon arborizations evenly fill the tectal neuropil. (B) Despite severely deformed eyes (see Figure 19 F for reference) *froggy* mutants form a remarkably normal retinotectal projection. Both dorsal (arrows) and ventral (arrowheads) axon tracts and tectal lobes are visible, and axon arborizations fill a smooth region of tectal neuropil. (C-D) Transmitted light channel shows tissue placement of gross morphological structures including eye, lens, brain in siblings and mutants. (E-F) TOPRO3 nuclear staining shows presence and placement of cells in sibling and mutant larvae. All images are maximum intensity projections, dorsal views, anterior up.

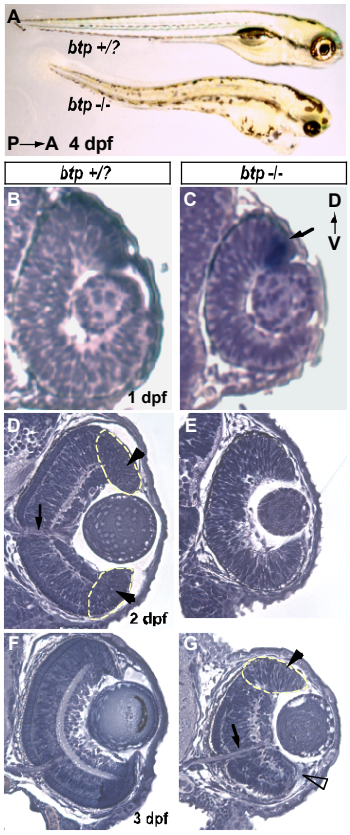


Figure 21. *btp* mutants are morphologically abnormal, and have delayed retinal development. (A) Four dpf *btp* mutants have a small eye (visible by two dpf), and smaller body (visible by 2.5 dpf). They develop a heart edema, never inflate their swimbladder, have fewer melanophores at two dpf, and display a visual background adaptation (VBA) phenotype at four dpf (see text for explanation). Mutants have a variably curled tail—curled up or down. Mutants also display a spontaneously twitchy seizure-like phenotype, observed beginning at three dpf. (B-C) At one dpf, prior to differentiation of retinal cell types, the *btp* mutant eye (C) is small in comparison to siblings (B), yet overall morphology, and appearance of individual cells is unaffected. These one dpf mutant and sibling mutants were sorted by their *in situ* phenotype, and this staining is visible in the dorsal retina in sections (arrow in C). (D-E) At two dpf, sibling retinas (D) contain numerous differentiated cell types that have organized into retinal layers. *btp* mutant retinas (E) appear significantly delayed. They have smaller neural retina and lens domains, and do not show any differentiated cell types, retinal layers or optic nerve. (F-G) At three dpf, retinal layers are significantly more organized in siblings (F), and lens has differentiated. Mutant retinas now show many differentiated cell types, organized retinal layers, and an optic nerve is visible. However these three dpf retinas appear almost a full day delayed, and differentiation of lens cells also appears delayed in mutants [compare mutant three dpf retina and lens (G) with those of the two dpf sibling (D)]. Additionally, the ventral CMZ zone, outlined in yellow, appears absent in *btp* mutants (again compare G with D). (A) Live embryos, (B-G) 12.5 µM transverse plastic sections, stained with Toluidine Blue.

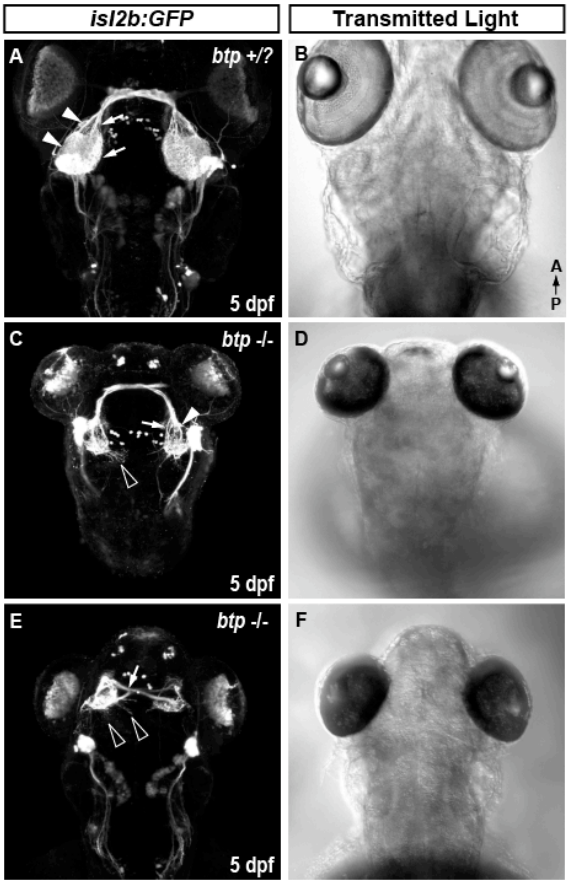
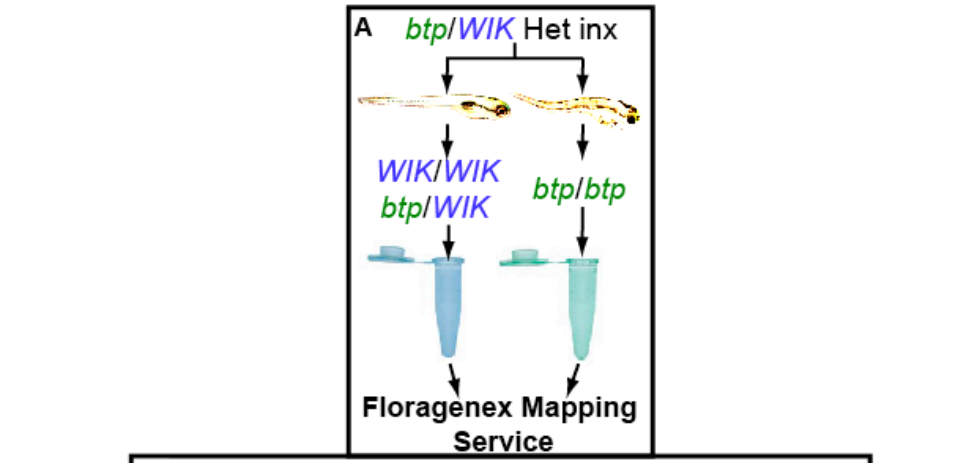


Figure 22. *btp* is necessary for retinotectal axon projections. The *isl2b:GFP* transgene marks retinal ganglion cells and their axon projections with GFP (as well as several other cell populations, including trigeminal neurons, and Rohon Beard cells). I incrossed *btp* +/-; *isl2B:GFP* embryos, and fixed and stained larvae at five dpf for GFP. (A-B) Robust retinotectal axon projections in sibling embryos (A) display clear dorsal (medial) and ventral (lateral) optic tracts and tectal lobes (arrows mark left dorsal tract and tectal lobe, arrowheads mark ventral tract and lobe). Axon arborizations evenly fill the tectal neuropil. (C, E) Axon projections in *btp* mutants are more sparse, as expected from the smaller retinas and smaller number of retinal ganglion cells. Dorsal and ventral tracts are apparent (arrow and arrowhead for dorsal and ventral respectively in C). Axon projections appear to arborize primarily on the posterior tectum, leaving the anterior tectum somewhat devoid of arborizations. Additionally, some axons project beyond the normal arborization zones in *btp* mutants and travel medially across the brain toward the opposite tectum, or posteriorly (open arrowheads in C and E). Finally, in some cases axons misproject ipsilaterally and innervate the ipsilateral tectum (arrow in E), leaving the contralateral tectum with very sparse projections. (B, D, F) Transmitted light channel shows the location of eyes and appearance of retinas and lenses in sibling and mutant embryos. All images are maximum intensity projections, dorsal views, anterior up.



C

RAD tag #	RAD Tag Seq	Counts in wt	Counts in mut	Chrom	Position in zv8
FGX_00023	TGCAGGAGATTCAGCAAATAATGAACTAAAAACGAGGATCATTT	14	0	2	42004412
FGX_00024	TGCAGGGGATGATGGTTGGGTAGCAAAGCTTGTACAGCAGAGAC	15	0	2	42226700
FGX_00025	TGCAGGCCAGCAGGAGGCTGGTGGAGAAGTTAAAAGCTCCTTTT	12	0	2	42386318
FGX_00026	TGCAGGTTTGAACTGCTGTGCCGCCTTATGTTGTTTTATGCTGT	12	0	2	42787647
FGX_00027	TGCAGGTAACTAGACAAAAAAAAGGGAAGCCCTAACGCATGTCC	7	0	2	43373134
FGX_00028	TGCAGGCTAGTGCAGAAACTCCATTGAAAATACTGGGGTTAAAT	12	0	2	43374171
FGX_00029	TGCAGGGACATAACAGAAAAAAATCAACATAGACTCATTCTGAA	7	0	2	43491243
FGX_00030	TGCAGGTATCCTAAACAATCACACATGGATATTTTCCATTCAGT	14	0	2	44249774
FGX_00031	TGCAGGATACCAGCAACACACGGGACACTCCTCAAAGCAAAGTA	14	0	2	44463089
FGX_00032	TGCAGGGGGCAGTAAAAATATTAACTCTGAACAGTTGACAATGT	17	0	2	44472374
FGX_00033	TGCAGGATACTGTACAGACTTTACAGTATATTTAGTACCCATCC	7	0	2	45661698
FGX_00034	TGCAGGTCTGCCACAGGCTATCAGCCATCTGGGCTTTTATGACG	13	0	2	45772564
FGX_00035	TGCAGGTCTGAAGCAGACTAAACTACACCATCTGAGGCCAGTTT	13	0	2	45787951
FGX_00036	TGCAGGATAATATCAGGCCTTGGTGAAAGGCTGGTTATGTTACC	14	0	2	45787993

Figure 23. The *btp* mutation is linked to chromosome 2. (A) *WIK* map cross strain DNA depicted in blue. Tübingen strain screen DNA (linked to the *btp* mutation) depicted in green. I incrossed *btp/WIK* map cross heterozygous adults, and separated *WIK/WIK*, *btp/WIK*, and *btp/btp* offspring into sibling and mutant pools according to four dpf phenotypes. I prepared DNA from each pool and sent it to Floragenex for mapping. (B) Floragenex calculated the average number of WIK-linked markers for each linkage group, and found a higher incidence of *WIK*-linked markers on chromosome two in the sibling pool compared to the mutant pool, significantly higher than the number expected by chance (red dotted line). This indicates a region of Tübingen-enriched DNA in the mutant pool, markers linked to the *bigtop* mutation, on chromosome two. (C) Analysis of Floragenex RAD tags on chromosome two identified a four megabase region between 42-46 million bases (in Sanger zebrafish genome version zv8) that is completely linked to the *bigtop* mutation, as indicated by the fact that these markers were only observed in the sibling DNA pool containing *WIK* DNA, and never in the mutant pool, containing only *btp* DNA in this region.

Table 1: Genes located in the critical region of chromosome two identified by Floragenex as completely linked to the *bigtop* mutation. In the zv9 reference genome, 92 annotated genes are located between the RAD tag markers identified by Floragenex as completely linked to the *bigtop* mutation. Many of these genes have known expression in the retinas of zebrafish and other species. Yes – retinal expression observed at some time point. Unknown – expression of this gene has not been studied, or expression data available, with no observed expression in the retina. N/A – no homology information for this gene in other species, or no known orthologs. Gene information obtained from Ensembl: http://May2012.archive.ensembl.org/Danio_rerio/Info/Index. Expression data obtained from Bgee: a database for Gene Expression Evolution (Bastian, 2008).

Genes in the region 43000000-47000000 of chromosome 2:						
	Gene		Biotype:	Location:	Expression in Zebrafish Retina	Expression in Other Spp. Retina
1	EFR3A(2 of 2)	ENSDARG00000005163	protein_coding	42973773-43027438 bp	Unknown	Yes
2	oc90	ENSDARG00000001721	protein_coding	43043670-43061471 bp	Yes	Yes
4	HHLA1	ENSDARG00000087842	protein_coding	43081596-43082550 bp	Unknown	Unknown
6	kcnq3	ENSDARG00000060085	protein_coding	43102630-43184873 bp	Unknown	Yes
7	lrrc6	ENSDARG00000053318	protein_coding	43199355-43225067 bp	Unknown	Yes
8	ftr13	ENSDARG00000079537	protein_coding	43229480-43232048 bp	Unknown	N/A
9	ftr14	ENSDARG00000053293	protein_coding	43232265-43236818 bp	Yes	N/A
10	ftr15	ENSDARG00000074118	protein_coding	43245068-43252476 bp	Unknown	N/A
11		ENSDARG00000023217	protein_coding	43256633-43297713 bp	Unknown	Unknown
12	pard3	ENSDARG00000010583	protein_coding	43306095-43490366 bp	Yes	Yes
14	nrp1b	ENSDARG00000027290	protein_coding	43570359-43633838 bp	Yes	Yes
15	itgb1b.2	ENSDARG00000022689	protein_coding	43670284-43684517 bp	Unknown	N/A
16	itgb1b.1	ENSDARG00000053232	protein_coding	43687135-43704034 bp	Unknown	N/A
17	EPC1(1 of 2)	ENSDARG00000060054	protein_coding	43717977-43737245 bp	Unknown	Yes
18	CU693495.1	ENSDARG00000088955	protein_coding	43739588-43750519 bp	Unknown	N/A
19	CABZ01071036.1	ENSDARG00000088800	protein_coding	43757513-43758022 bp	Unknown	N/A
20	kif5ba	ENSDARG00000074131	protein_coding	43765423-43801060 bp	Unknown	Yes
21	ftr16	ENSDARG00000032043	protein_coding	43804098-43806647 bp	Unknown	N/A
22	ARHGAP12 (2 of 2)	ENSDARG00000008548	protein_coding	43810722-43858719 bp	Yes	Yes
23	ZEB1 (2 of 2)	ENSDARG00000016788	protein_coding	43868731-43913416 bp	Yes	Yes
24	CR848751.1	ENSDARG00000090699	protein_coding	43926076-43934272 bp	Unknown	N/A
25	gbp3	ENSDARG00000003244	protein_coding	43955869-43980375 bp	Unknown	Yes
26	CARD8	ENSDARG00000068431	protein_coding	43981962-44016911 bp	Unknown	Yes
27	si:ch211-195h23.4	ENSDARG00000093337	protein_coding	44002426-44004096 bp	Unknown	N/A
28	kirrelb	ENSDARG00000056998	protein_coding	44023127-44100277 bp	Unknown	Yes
29	cadm3	ENSDARG00000057013	protein_coding	44105656-44245114 bp	Yes	Yes
30	sdhc	ENSDARG00000038608	protein_coding	44254153-44261341 bp	Yes	Yes
31	atp1a2a	ENSDARG00000010472	protein_coding	44264576-44317038 bp	Yes	Yes

32	mpz	ENSDARG00000038609	protein_coding	44320983-44344410 bp	Yes	Yes
33	LIG1	ENSDARG00000060041	protein_coding	44362777-44405822 bp	Yes	Yes
34	zgc:152670	ENSDARG00000017038	protein_coding	44409974-44424444 bp	Unknown	N/A
35	si:ch211-265g22.4	ENSDARG00000076512	protein_coding	44427193-44432915 bp	Unknown	N/A
36	TMEM55B (1 of 2)	ENSDARG00000038615	protein_coding	44444676-44460112 bp	Unknown	Yes
37	C2HXorf41	ENSDARG00000038612	protein_coding	44462271-44467626 bp	Unknown	N/A
38	KCNAB1 (3 of 3)	ENSDARG00000040741	protein_coding	44488110-44572253 bp	Yes	Yes
39	PASK	ENSDARG00000075008	protein_coding	44573825-44607370 bp	Yes	Yes
40	si:dkeyp-94h10.3	ENSDARG00000094463	protein_coding	44584357-44585882 bp	Unknown	Unknown
41	MTERFD2	ENSDARG00000079124	protein_coding	44607413-44618819 bp	Yes	Yes
42	GPR148	ENSDARG00000060024	protein_coding	44623829-44625766 bp	Unknown	Unknown
43	BX323035.2	ENSDARG00000091424	protein_coding	44627715-44628827 bp	Unknown	N/A
45	klhl24	ENSDARG00000021739	protein_coding	44632701-44665956 bp	Unknown	Yes
46	YEATS2	ENSDARG00000078767	protein_coding	44677192-44756745 bp	Unknown	Yes
47	MAP6D1	ENSDARG00000079777	protein_coding	44759069-44782214 bp	Unknown	Yes
48	C2H10orf129	ENSDARG00000004880	protein_coding	44787051-44808224 bp	Unknown	Unknown
49	ncapd2	ENSDARG00000005058	protein_coding	44811404-44839093 bp	Yes	Yes
50	snoU85	ENSDARG00000083325	snoRNA	44824583-44824876 bp	Unknown	Unknown
51	snoU85	ENSDARG00000081126	snoRNA	44835776-44836096 bp	Unknown	Unknown
52	si:ch211-224b1.4	ENSDARG00000068021	protein_coding	44842735-44984354 bp	Yes	N/A
53	si:ch211-224b1.5	ENSDARG00000092428	protein_coding	44985300-44986177 bp	Unknown	N/A
54	camk2n1a	ENSDARG00000025855	protein_coding	45004897-45007595 bp	Yes	Yes
55	mul1a	ENSDARG00000021398	protein_coding	45022377-45033052 bp	Unknown	Yes
56	alg3	ENSDARG00000053155	protein_coding	45034221-45042845 bp	Yes	Yes
57	vwa5b2	ENSDARG00000075886	protein_coding	45046044-45083205 bp	Unknown	Yes
58	si:dkey-76d14.1	ENSDARG00000053158	protein_coding	45110791-45119741 bp	Unknown	N/A
59	si:dkey-76d14.2	ENSDARG00000093458	protein_coding	45129867-45131943 bp	Unknown	N/A
60	CHRNG	ENSDARG00000086647	protein_coding	45143065-45158422 bp	Unknown	Unknown
61	eif4e2	ENSDARG00000038585	protein_coding	45178320-45197119 bp	Yes	Yes
62	CAPN10	ENSDARG00000076634	protein_coding	45200971-45216123 bp	Unknown	Yes
63	CU929150.1	ENSDARG00000038587	protein_coding	45221499-45222824 bp	Unknown	N/A

64	CCL20 (2 of 2)	ENSDARG00000093754	protein_coding	45241704-45243634 bp	Unknown	N/A
65	dre-mir-181b-1	ENSDARG00000081145	miRNA	45252630-45252738 bp	Unknown	N/A
66	CU571393.1	ENSDARG00000081893	miRNA	45255036-45255102 bp	Unknown	N/A
67	CAMSAP2 (2 of 2)	ENSDARG00000059965	protein_coding	45346799-45431744 bp	Unknown	N/A
70	CH211-141H20.6	ENSDARG00000076809	protein_coding	45387428-45392266 bp	Unknown	N/A
71	wdr47	ENSDARG00000078136	protein_coding	45437773-45478472 bp	Unknown	Yes
72	si:ch211-66k16.20	ENSDARG00000092789	processed_transcript	45487179-45503631 bp	Unknown	N/A
73	BX005331.2	ENSDARG00000091280	protein_coding	45497125-45497715 bp	Unknown	N/A
74	si:ch211-66k16.2	ENSDARG00000095634	protein_coding	45510718-45512008 bp	Unknown	N/A
75	si:ch211-66k16.24	ENSDARG00000094989	protein_coding	45516473-45517314 bp	Unknown	N/A
76	BX005331.1	ENSDARG00000088423	protein_coding	45535612-45542837 bp	Unknown	N/A
77	gpsm2	ENSDARG00000017311	protein_coding	45546372-45566044 bp	Yes	Yes
78	si:dkeyp-51f11.3	ENSDARG00000094414	protein_coding	45566799-45593275 bp	Unknown	N/A
79	fndc7	ENSDARG00000077096	protein_coding	45604230-45623042 bp	Unknown	Unknown
80		ENSDARG00000092552	protein_coding	45649123-45653991 bp	Unknown	N/A
81		ENSDARG00000092469	protein_coding	45675289-45682103 bp	Unknown	N/A
82	si:dkeyp-51f11.9	ENSDARG00000092470	protein_coding	45691374-45694499 bp	Unknown	N/A
83		ENSDARG00000092471	protein_coding	45698462-45707715 bp	Unknown	N/A
84	prpf38b	ENSDARG00000092472	protein_coding	45710634-45719285 bp	Yes	Yes
85	fam102ba	ENSDARG00000092473	protein_coding	45720833-45746468 bp	Unknown	Yes
86	CU467828.1	ENSDARG00000092474	protein_coding	45752083-45931456 bp	Unknown	N/A
87	si:ch211-170p16.1	ENSDARG00000092475	protein_coding	46050492-46053459 bp	Unknown	N/A
88	itgb1b	ENSDARG00000092476	protein_coding	46095866-46102453 bp	Yes	Yes
89	GPC6	ENSDARG00000092477	protein_coding	46421273-46423420 bp	Yes	Yes
90	gpc1	ENSDARG00000092478	protein_coding	46430883-46442745 bp	Unknown	Yes
91	EPHB1	ENSDARG00000092479	protein_coding	46498776-46805910 bp	Unknown	Yes
92	NPC1L1	ENSDARG00000092480	protein_coding	46965059-46987692 bp	Unknown	Yes

CHAPTER 4

DISCUSSION

Summary

The research presented in my thesis has clarified the mechanisms of eye patterning and topographic retinotectal projections by focusing on the dorsal-ventral (D-V) axis of the retina. My experiments have used the embryonic zebrafish, and analyzed polarized dorsal-ventral gene expression patterns during retinal morphogenesis, tested the roles of known D-V axis retinal genes, and screened for novel gene involved in specifying D-V retinal cell fate. Development of continuous topographic neural maps, such as the retinotopic map in the visual system, requires tissue patterning of both source and target tissues (McLaughlin et al., 2003; Harada et al., 2007). This tissue patterning is generally achieved via a genetic cascade involving many different genes, and several different gene classes, including secreted factors, transcription factors, and axon guidance ligands and receptors, which ultimately direct the precise targeting of neurites. Accurate neurite targeting is necessary for normal nervous system function.

In my work outlined in Chapter 2 I characterized the early events of dorsal retinal patterning and determined the required roles of candidate morphogens for initiation of dorsal polarity. It is known that both morphogens and transcription factors play roles in specifying dorsal retinal polarity, yet the genes necessary for initiating dorsal character had not been identified prior to my work. In particular, I predicted that the initiation

factor would be an extraocular signal that could diffuse into the prospective dorsal retinal region in order to initiate dorsal fate. Whereas the Bmp family morphogen gene *gdf6a* can regulate initiation of many D-V patterning genes (French et al., 2009), it was not known whether this gene was acting extraocularly, or within the retinal field. My work shows that dorsal retina is initiated by *gdf6a* expression arising from extraocular ectoderm overlying the dorsolateral optic vesicle leaflet at 11-12 hpf. Thus, I have identified the tissue source and time of action of at least one extraocular factor critical for dorsal retinal initiation, though my results indicate that there may be at least one other necessary factor. My work also uncovered a novel role of the *bmp2b* gene in dorsal retinal initiation, acting upstream of *gdf6a* in this process, likely to form the non-neural ectoderm. This is the critical tissue for initiation. *bmp2b* mutants, which lack extraocular ectoderm tissue and the extraocular *gdf6a* expression therein, never initiate dorsal fate. In summary, my work outlined in the second chapter is focused on the mechanism of dorsal retina fate initiation. While specific molecules involved may vary between species, I believe that the basic principles are likely conserved across vertebrates.

In research described in Chapter 3 I performed a forward genetic screen to identify novel genes with required roles in dorsal-ventral retinal patterning. I generated, recovered, cloned and characterized a novel mutant, which I named *bigtop* due to the expansion of dorsal retinal patterning genes. My characterization of the *bigtop* mutant shows that this gene is necessary for the maintenance of D-V retinal patterning. Dorsal retinal patterning genes are initiated normally in these mutants, but their expression expands following initiation, at 16-18 hpf, and this domain expansion is persistent at 44 hpf. Similarly, ventral retinal patterning genes are initiated normally, but restrict to

smaller domains and decreased staining intensity by 24 hpf. These mutants also have delayed eye development and altered retinotectal axon projections. Contracting with the company Floragenex, I mapped this mutation to a four-megabase region on chromosome two; however, I have not yet been successful with cloning the *bigtop* gene.

In discussion of my thesis work, I would like to put my research in the perspective of the field of D-V eye patterning research. I would also like to suggest future avenues for ongoing research. In doing so, I will highlight areas where my research identified novel findings in this field. I will also point out potential caveats to my experiments, or unsuccessful areas of my research. These research downfalls often bring up alternative approaches and new questions that could be followed in the future.

Dorsal Retinal Initiation

Dorsal initiation tissue

My work in chapter two demonstrated novel relevance for a basic tissue patterning strategy—namely that patterning within a tissue can be initiated due to a signal arising from a neighboring tissue source. Several lines of evidence in my work suggest that dorsal retina is initiated by the morphogen Gdf6a (and possibly also Bmp2b) diffusing from the extraocular ectoderm, which is directly adjacent to the dorsolateral optic vesicle during optic vesicle evagination. However, I was not successful at ablating extraocular ectoderm tissue, and observing loss of initiation of dorsal retinal fate. This would determine that this tissue is necessary for initiation of dorsal patterning genes, and that phenotypes seen in mutants are indeed due to gene expression in this region. This classic embryological loss of function perturbation would be difficult due to the timing

(10-11 hpf), and the need to eliminate a large swath of ectoderm in order to eliminate *gdf6a* (and *bmp2b*) expression (see *gdf6a* expression domain in Chapter 2, Figure 15 A). My attempts at this tissue ablation experiment led to rapid tissue healing, and no loss of expression of dorsal markers. It is possible that neighboring cells expressing *gdf6a* migrate to populate the wound site, therefore bringing Gdf6a back to the correct location for dorsal initiation. Novel techniques for removing ectoderm overlying dorsolateral optic vesicle, and subsequently inhibiting wound healing may be necessary for this experiment.

An alternative approach to manual removal of the extraocular ectoderm retinal initiation tissue would be causing a genetic loss of this tissue, or a conditional loss of *gdf6a*, only within this tissue. I showed using *bmp2b* null mutants that genetic loss of all non-neural ectoderm leads to loss of dorsal retinal fate, as *bmp2b* is required for specification of this tissue during early development (Nguyen et al., 1998). However, this elimination is not specifically eliminating non-neural ectoderm overlying the optic vesicle at 10-12 hpf. Other more targeted genetic tissue ablations may be able to test the elimination of only extraocular ectoderm more specifically. Alternatively conditional loss of *gdf6a* specifically in non-neural ectoderm may also be possible, as conditional gene targeting strategies become more widely available in zebrafish.

French et al. (2009) showed that early ectopic expression of *gdf6a* driven within the optic vesicle by the *rx3* eye field promoter could drive initiation and domain expansion of dorsal retinal markers and reduction of ventral markers. Although I attempted several similarly themed gain-of-function experiments to test the sufficiency of *gdf6a* specifically from the extraocular ectoderm tissue for dorsal initiation, these experiments were unsuccessful or difficult to interpret. Due to the diffusible nature of the

morphogens required, widespread dispersion from ectoderm could be taking the place of morphogens normally emanating from another tissue source. These experiments may also be complicated by "cross-talk" between the multiple *bmp* gene family members, whereby overexpression allows one *bmp* to play the role another normally plays in the wild type condition. Regardless, potential experiments attempted or discussed in our lab included extraocular implantation of an agarose bead soaked in recombinant Gdf6a, or misexpression of *gdf6a* from an extraocular promoter (such as a lens placode promoter) to rescue dorsal initiation in *gdf6a* mutants or morphants. In my attempts, I was not able to find a lens placode promoter that drove expression of downstream genes early enough to be relevant for dorsal retina initiation (*pitx1* and *prox3* promoters attempted). Furthermore, while I was able to drive widespread expression of *gdf6a* throughout the enveloping layer, under control of the *krt4* promoter, this caused loss of eye structures in both mutants and siblings, as has been seen previously following global overexpression of *gdf6a*. Therefore results were difficult to interpret because both mutants and siblings showed loss of expression of dorsal markers, along with significant loss of the eye field, as assayed by the *rx3* homeobox transcription factor eye field marker. Due to the caveats of overexpression experiments outlined above, I would not suggest continued attempts at these gain-of-function experiments as avenues for future research.

I hypothesize that extraocular initiation of dorsal fate is conserved across evolution, however extraocular initiation signals have not been identified in other species. Future experiments in mouse could test the required tissues for dorsal fate initiation. It is possible that *gdf6a* homologs are also acting as the extraocular initiator in other species, or that *Bmp4* for example, is playing the role of the extraocular initiator in mouse.

Timing of dorsal retinal initiation

I used the selective pharmacological BMP inhibitor LDN 193189 (Cannon et al., 2010) to show that Bmps are acting to initiate dorsal retina between 9.5 and 13 hpf, prior to the expression of *bmp* genes within the optic vesicle. This experiment has never been done previously, and is especially exciting because past pharmacological attempts to inhibit dorsal retinal initiation using Bmp inhibitors, such as with Dorsomorphin [my unpublished results and (French et al., 2009)], have been unsuccessful, even when high doses of drug are used. Further testing with this pharmacological inhibitor could tell me more about dorsal fate initiation. For example, *bmp2b* and *gdf6a* mutants have a small amount of dorsal character remaining. Both mutants initiate expression of *tbx2a* in a smaller domain than normal, and *gdf6a* mutants initiate a small domain of *tbx4* at 16 hpf. If this residual dorsal character were lost following treatment with LDN from 9.5-13 hpf, this would indicate that *bmp2b* (or another *bmp*) is acting in conjunction with the *gdf6a* from extraocular ectoderm to initiate dorsal retinal fate. However, if this small amount of dorsal character still exists following 9.5-13 hpf LDN treatment (as assayed by *tbx2a* and *tbx4* still expressed in a small domain as in *gdf6a* mutants), this suggests that the only role of *bmp2b* in dorsal retinal initiation is its role in formation of non-neural ectoderm, and that its extraocular expression during initiation time points (10-12 hpf) has no necessary function for initiating dorsal fate. Instead, another non-*bmp* gene, such as the *pbx* or *meis1* homeodomain transcription factors for example (French et al., 2007; Erickson et al., 2010), may be contributing this residual dorsal fate to the mostly ventralized retinas of *gdf6a* mutants.

Additionally, I could also use the LDN drug to analyze whether dorsal fate initiation is a discrete early event, or whether it can also happen later in development. *gdf6a* is expressed extraocularly in a dynamically changing domain that covers the domain of dorsal patterning markers within the optic cup during morphogenesis from 11-15 hpf. After 15 hpf, expression of *gdf6a* in the ectoderm is slowly downregulated, while expression within the prospective dorsal retina is upregulated, and maintained in a restricted domain at 24 hpf. This expression pattern indicates that *gdf6a* initiation of dorsal retina may be an event that can occur over time, and does not need to happen as early as it does in the wild type case. I could test this hypothesis by treating with LDN from 9.5-13 hpf, which I know blocks dorsal initiation (Chapter 2, Figure 9), then washing it off and fixing several hours later. If initiation still occurs, then it is likely a plastic event that can occur over time. If this is the case, it would be interesting to test how late in development this is true. For example, could dorsal retinal patterning still be initiated by *bmp* signaling turned on at 24 hpf?

Role of Gdf6a in dorsal retinal initiation

gdf6a is critical for initiating and maintaining dorsal retinal fate in zebrafish (French et al., 2009; Gosse and Baier, 2009). However, contrary to what was seen using *gdf6a* morpholinos (French et al., 2009), I observed that a small amount of dorsal retinal character is still present in *gdf6a* mutants (Chapter 2, Figure 12 and Figure 13). Ventral retinal genes only expand to cover ~90% of the retina at 24 hpf, and a few dorsal retinal genes are initiated and/or maintained in small domains. It is unclear why this discrepancy between the morpholino and mutant data exists, and especially unclear why morphants would have a stronger phenotype. Possibly this morpholino is also blocking other *bmp*

genes nonspecifically. Alternatively, minor variations in experimental protocols may be causing this, for example time of fixation, or length of time *in situ* staining is allowed to develop. If I believe that the mutant data is correct, and a small amount of dorsal character still exists following elimination of *gdf6a* function, it would be interesting to know what is specifying this residual dorsal character. I hypothesize in chapter two that *bmp2b* expressed in the extraocular ectoderm at initiation time points may be acting in conjunction with *gdf6a* to initiate dorsal retina. Alternatively other gene families may be contributing to the small amount of dorsal character still existing in *gdf6a* mutants (French et al., 2007; Erickson et al., 2010).

In *gdf6a* mutants all retinal ganglion cells map to the dorsal optic tectum, leaving the ventral tectum empty, without any axon innervation (Muto et al., 2005). Interestingly, however, axons still sort with D-V retinotectal topography in *gdf6a* mutants, within the dorsal half of the optic tectum (Gosse and Baier, 2009). How is this topographic mapping of axon projections occurring in the *gdf6a* mutant while the large majority of the eye is patterned with ventral markers? This mapping could be due to the very small amount of residual dorsal fate in *gdf6a* mutants, or it could be due to other D-V patterning signals not yet identified that are not regulated by *gdf6a*. Alternatively, this mapping could be due to axon-axon interactions or neural activity (McLaughlin and O'Leary, 2005). Future experiments could test if this topographic mapping phenotype is dependent on cell adhesion molecules, neural activity in response to light, or spontaneous retinal waves.

Finally, while *gdf6a* is clearly important for specifying dorsal retinal fate in zebrafish, the role of this gene in axial patterning in other species is not yet known. Due to the similar expression patterns and known phenotypes of loss of function in other

species, I hypothesize that *Gdf6* could have a very similar role in mouse and/or humans, as the extraocular initiator of dorsal fate. Yet the specific roles of this gene for D-V retinal patterning have not been tested in these species. This could be tested using conditional loss of function experiments in mouse.

Role of Bmp2b in dorsal retinal initiation

I found that *bmp2b* acts upstream of extraocular *gdf6a*: *bmp2b* mutants show abnormal extraocular expression of *gdf6a*, but extraocular *bmp2b* expression is normal in *gdf6a* mutants. However, *bmp2b* acts downstream of *gdf6a* in the retina: in *gdf6a* mutants, retinal expression of *bmp2b* is lost. Loss of retinal *bmp2b* in *gdf6a* mutants is contrary to what was seen in (Gosse and Baier, 2009), likely due to the accidental analysis of heterozygotes instead of mutants in this paper. Finally, retinal expression of *gdf6a* cannot be analyzed in *bmp2b* mutants, because these mutants die before *gdf6a* expression turns on within the retina. This placement of *bmp2b* and *gdf6a* in a signaling pathway had not been shown previously. I think that the primary role of extraocular *bmp2b* is in the specification of the extraocular ectoderm (Nguyen et al., 1998), the necessary tissue for expressing *gdf6a* to initiate dorsal fate. *bmp2b* mutants are strongly dorsalized at 12 hpf, and dead by 15 hpf. While I was able to analyze the initiation of a few patterning genes (*tbx2a, tbx5* and *pax2a*) in this mutant, I have not been able to test the effect of loss of *bmp2b* on later eye patterning genes. Additionally, I have not been able to rule out a later role for this gene, acting along with *gdf6a* in extraocular ectoderm during initiation time points. This could be done using conditional inactivation of *bmp2b*, and it may be necessary to do this in a *gdf6a* mutant background. I carried out experiments using caged *bmp2b* morpholinos (morpholinos activated only upon exposure

to UV light) to analyze loss of *bmp2b* function conditionally, following gastrulation. My caged morpholino experiments were able to recapitulate strong loss of *bmp2b* function when activated early, but I was not able to see a significant loss of dorsal markers when morpholinos were activated after six hpf. These experiments indicate that any role of *bmp2b* in dorsal fate initiation following gastrulation is minor, and secondary to the role of *gdf6a*. Therefore, if there is a secondary role of *bmp2b* acting in the extraocular ectoderm at 10-12 hpf, it may be necessary to test this role in the *gdf6a* background.

BMP2 in chick has been shown to act in dorsal gene maintenance, but not in initiation of dorsal retinal fate (Sakuta et al., 2006). Analysis of the function of *Bmp2* in mouse retinal development would require conditional inactivation of this gene in the retinal field and/or extraocular tissues, as global *Bmp2* knockouts die early in embryonic development (Zhang and Bradley, 1996).

Role of Bmp4 in dorsal retinal initiation

Bmp4 expression in dorsal retina is conserved across vertebrates. *Bmp4* has a critical role in specifying dorsal retinal fate in mouse (Murali et al., 2005; Behesti et al., 2006), yet the same role for this gene in other species has not been demonstrated. Gain-of-function experiments in *Xenopus* and chick show that misexpression of *bmp4* can lead to dorsalization of the ventral retina (Koshiba-Takeuchi et al., 2000; Sasagawa et al., 2002), but its role has not been tested using loss of function approaches in these species. In zebrafish from early to late time points, I have not yet been able to find any role of this gene in D-V patterning. It is possible that this gene has an as-yet unidentified paralog (e.g. *bmp4b*) in zebrafish that is acting redundantly with the known *bmp4* and masking the role of this gene. Or alternatively, *bmp4* could be acting redundantly with the other

known *bmps* expressed in dorsal retina. My unpublished results testing *bmp4*;*gdf6a* double mutants, and *bmp4*;*bmp2b* double mutants were not conclusive for uncovering an interacting or supporting role for this gene in dorsal initiation.

Dorsal Retina Maintenance and Refinement

Maintenance and refinement of gene expression

Dorsal-ventral retinal patterning is initiated early during development, during optic vesicle evagination, at 11-12 hpf. Intriguingly, all genes expressed in polarized patterns along the D-V axis early during development also maintain their expression over time. Why do dorsal initiation factors for example, maintain and refine their expression in the 24-48 hpf retina? Do these genes still have a role in retinal patterning at these late time points? Due to the continued expression of these genes, I hypothesize that they continue to be necessary for feedback control over the genes they regulate, thus maintaining the specification of tissues with their positional fate. In cases of retinal injury or retinal ganglion cell axon severing, the continued expression of these genes could be important for regeneration of tissues and/or axons. These hypotheses could be tested using conditional inactivation of these genes at late time points, potentially combined with injury and regeneration models.

Redundancy of gene function

Gene redundancy is a phenomenon where two or more genes can have fully or partially overlapping roles in a tissue. This is an important factor to consider in zebrafish research, due to the genome duplication that occurred during teleost evolution (Woods et al., 2000). Redundancy of gene function may be particularly prevalent in D-V retinal

patterning, as evidenced by the numerous members of gene families expressed in overlapping domains. For example, dorsally, at least six T-box family transcription factors (*tbx2a*, *2b*, *3*, *4*, *5a*, *5b*) are all expressed in overlapping patterns within the dorsal retina at 24 hpf. Additionally, at least five *bmp* family genes (*bmp2a, bmp2b*, *bmp4*, *bmp7b* and *gdf6a*) and two *ephrins* (*ephrinB1* and *ephrinB2a*) are also expressed in polarized patterns dorsally at this time (Thisse, 2001; Thisse, 2005; Shawi and Serluca, 2008). I hypothesize, due to the overlapping expression domains of many of these genes, that they may have completely or partially redundant roles in dorsal retina fate specification. One disadvantage of forward genetic screens is that they cannot recover mutations in redundant genes. Therefore, it may have been difficult to impossible to identify mutations in a single *tbx* gene for example with my screen. Additionally, the only other screen analyzing retinotectal topography found only two D-V mapping mutants, *nevermind* and *whocares* (Trowe et al., 1996). Could gene redundancy be a fundamental challenge to studying D-V retinal mapping and retinotectal topography? This may be the reason why many genes with polarized D-V expression patterns have no known function in D-V patterning to date. For example, *tbx5a* null zebrafish mutants have only minor retinal patterning phenotypes, and no defects in D-V topographic mapping [my unpublished results consistent with results using morpholinos in (French et al., 2009)]. I hypothesized that this is due to redundancy with the other *tbx* genes expressed in overlapping domains. Another example is the zebrafish *ephrinB2a* mutant. I was very interested to analyze retinotectal topography in this null mutant, found in the Sanger Tilling screen. *ephrinB2a* is one of only two *ephrins* that is robustly expressed in a broad gradient, high in the dorsal retina. However, I could not identify any retinotectal mapping

phenotypes in this mutant (our unpublished results). Additionally I attempted using a dominant negative *ephrinB2a*, and *ephrinB2a* morpholinos. My best interpretation of these negative results is that *ephrinB2a* is acting redundantly with *ephrinB1* or other unknown *eprhins* in dorsal retina. If redundancy is common in D-V retinal patterning, as I hypothesize, reverse genetic approaches may be more fruitful for uncovering the roles of D-V patterning genes. Targeted gene deletions or already available mutants could be combined to create double and triple gene mutants, which may reveal the overlapping functions of many dorsal patterning genes.

Formation of guidance molecule gradients

The retinotopic map is a continuous neural map, wherein neighboring retinal ganglion cell axons map to neighboring target zones. The mechanism for formation of such continuous neural maps is generally defined as *graded* expression of axon guidance receptors in the source tissue axons, responding to gradients of axon guidance ligands in the target. However, the mechanism for setting up these molecular gradients is not well defined. One long-term question of my research asks how expression of axon guidance receptors can be set up in a gradient, when the transcription factors regulating them are non-diffusible, and appear to be expressed in very precise domains, not in gradients.

Overlapping *tbx* transcription factor expression in the dorsal retina points to one potential answer to this question. Six *tbx* genes are expressed dorsally, with domain size roughly in the following order around the dorsal pole at 24 hpf: *tbx5a* < *tbx5b* < *tbx4* < *tbx2a* < *tbx3* < *tbx2b*. Therefore, *tbx5a* has the most refined domain, and *tbx2b* has the broadest domain. Expression appears precise, on or off within a cell rather than graded, and we know that transcription factors act within a cell and are not diffusible. How does

this apparently binary cue turn into a gradient of axon guidance receptor transcription? I hypothesized that the overlapping pattern of *tbxs* could create the "gradient" necessary for formation of a true gradient of axon guidance molecules. For example, the combinatorial expression of all *tbx* genes at the dorsal-most pole could lead to very high levels of axon guidance receptor transcription, whereas, further from the pole where only a few of *tbxs* are expressed, this combination leads to lower transcription. Perhaps mapping is initially more binary within D-V categories, and becomes graded only after neuronal activity begins, or via cell-cell communication. The topographic mapping of retinal axons has never been tested precisely enough to answer this question, however, it could be very interesting to pursue.

Precise quantification of D-V topographic mapping defects

Along with redundancy of gene function, discussed earlier, I hypothesize that some genes will have only minor roles in D-V topographic mapping. Due to the relatively crude state of techniques for analyzing D-V retinotectal topography, I think it is likely that some available D-V patterning mutants have subtle topographic mapping phenotypes that have not been identified in previous attempts. For example, zebrafish *tbx5a* mutants show altered expression of some D-V patterning markers, yet no D-V mapping phenotype has been identified in these mutants (my unpublished results). Similarly, transgenic repression of *wnt* signaling causes loss of dorsal patterning markers (Veien et al., 2008), yet no observed defect in D-V retinotectal mapping (my unpublished results). Current methods for quantifying retinotectal topography are limited in several ways. Lipophilic dye injections are not reproducible across animals, precluding accurate quantification. They are also time-consuming and technically challenging. Finally, they

label large groups of cells, so targeting of individual axons cannot be assayed, and less severe phenotypes will likely be missed. Alternatively, individual cells can be labeled with dyes. However, labeling one cell per retina is labor intensive, and it is impossible to analyze the relative projections of multiple cells. It would be very advantageous to devise a new method for analyzing retinotectal topography that is more precise and quantifiable. I propose using Brainbow transgenes (driven transiently from the *isl2B* promoter) to label a small subset of retinal ganglion cells and their axon projections to the tectum. This approach would allow quantification of topographic targeting of multiple RGCs per animal (including the relative targeting of many cells), and require no manipulation of individual animals, thus providing increased sensitivity and ease of usability for visualizing and quantifying targeting defects. Alternatively to the Brainbow approach, stable transgenes that label small subsets of dorsal axons, ventral axons, or both, driven by D-V promoters could be used; however, these types of transgenics have thus far been difficult to generate in my attempts. A future screen for D-V mapping mutants could use such a D-V transgenic to analyze topographic tectal mapping in a less labor intensive way than the past screen employing dye injections (Trowe et al., 1996).

bigtop cloning

bigtop is a recessive mutation identified in my forward genetic screen, necessary for maintenance of D-V retinal fate. My attempts to clone the gene mutated in *bigtop*^{zc52} mutants using RNA sequencing have been unsuccessful thus far. I hoped to identify a novel nonsense mutation in mutant mRNA, leading to an early stop codon, and other labs have been successful in using this approach. Alternatively, a missense mutation, a SNP leading to a nonsynonymous amino acid substitution critical to protein function, could

also be informative. Missense mutations are expected throughout the genome, however, and would require significant testing to verify that the observed amino acid change caused the mutant phenotype observed. Therefore, the most interesting missense mutations would be in candidate genes for my mutation, within the critical region identified by Floragenex as linked to *bigtop*. My lack of success with the RNA sequencing approach to cloning *bigtop* could be due to several different factors. Restricted or downregulated mRNA expression of the mutant gene at the time point analyzed would lead to a lack of sequence data for the mutated gene. Alternatively, maternal mRNA could "contaminate" the mutant sample by introducing wild type mRNA.

Nonfunctional mRNAs, such as those with early stop codons, can also be eliminated by nonsense-mediated decay; therefore, I may expect sequence data for the mutated gene to be absent or significantly lower in the mutant sample. I analyzed my RNA sequencing data for nonsense mediated decay of transcripts, yet I was not able to find any obvious candidates using this approach.

Within the four megabase genomic region identified by Floragenex, *ephB1*, a member of the receptor tyrosine kinase family of axon guidance receptors, is currently my primary candidate for the *bigtop* gene. While the RNA sequencing data has not uncovered a novel missense or nonsense mutation in this gene, this could be due to the caveats described above. Alternatively, *bigtop* could be a mutation in a regulatory region of this gene. I would not expect to uncover regulatory DNA mutations via RNA sequencing, because this method only analyzes expressed mRNAs. I will continue with

experiments for ruling out this candidate gene before moving on to other gene cloning approaches.

Genes peripherally related to D-V patterning

The unbiased nature of forward genetic screens, while ideal for identifying genes novel genes involved in a pathway, can also be problematic, because recovered mutants may only be distantly related to the process of interest. For example, the second mutation I recovered from my F3 screen, *froggy*^{zc54} exhibited grossly disrupted eye development and rapid retinal decay, a fully penetrant phenotype at three dpf. However, at 24 hpf, this mutation resulted in a partially penetrant loss of *vax2* phenotype. I did not pursue this mutant beyond initial characterization of gross morphology and retinal sections, because I thought the phenotypes observed were unlikely to be due to retinal patterning. Rather I hypothesized the early phenotypes were secondary to the impending retinal decay.

Conclusions and Outlook

In summary, my research addressed a fundamental question in developmental neurobiology: how does a tissue such as the retina acquire positional coordinates via tissue patterning? This cellular "chemospecificity code" within the retina is necessary for accurate expression of gradients of axon guidance molecules, which can then guide axons to their appropriate synaptic target cells. I studied tissue patterning in the D-V axis of the zebrafish retina. I showed that initiation of D-V retinal patterning requires an extraocular signal, namely Gdf6a, arising from extraocular ectoderm. I also identified a novel gene necessary for patterning the D-V retinal axis in zebrafish. These experiments advanced the field of D-V retinal patterning, and my understanding of eye development in general.

In the visual system, accurate axon projections are essential for normal visual function. Therefore, these studies have broad relevance because neurite guidance defects during development can cause neurological diseases. Development of the visual system is very well conserved across vertebrates; therefore my studies have relevance to human retinal development. An understanding of the systems that underlie the patterning and formation of topographic projections of the eye could lead to treatments for developmental eye diseases such as coloboma and microphthalmia, as well as ways to manipulate the system to allow regeneration in non-regenerative adult CNS tissues following injury.

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